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# AN ASSAY METHOD FOR ANTIDIURETIC HORMONE BASED ON A MORE SPECIFIC RESPONSE INDEX[1]

JOHN D. CRAWFORD AND BARBARA PINKHAM

*Department of Pediatrics, Harvard Medical School and Children's Medical Service, Massachusetts General Hospital, Boston, Massachusetts*

## INTRODUCTION

NUMEROUS methods have been proposed over the past 25 years for the assay of posterior pituitary antidiuretic hormone (A.D.H.). Many of these have recently been reviewed by Stein (1952). Most of the methods can be classified in two categories according to the type of index of response used. One category includes the methods in which increment in urinary excretion of an individual solute, usually chloride, has been related to the A.D.H. content of the administered material (Anselmino, 1932; Ham, 1942, 1943; Ralli, 1950). The second category consists of methods where the index of response is change in rate of urine flow (Gibbs, 1930; Burn, 1931; Theobald, 1934; Gilman, 1937; Walker, 1939; Robinson, 1940; Jeffers, 1942; Birnie, 1950; Ames, 1950; Stein, 1952; Ginsburg, 1953; Dicker, 1953).

Neither of these response indices can be considered specific. Increments in urinary solute excretion, particularly chloride and sodium, are only indirectly related to A.D.H. action (Leaf, 1953). Such increments may fail to occur under certain circumstances when A.D.H. is administered (Sinclair-Smith, 1950; Black, 1951; Murphy, 1951; White, 1951; Chalmers, 1951; Talbot, 1952; Nelson, 1952); they can be induced in the absence of change

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in A.D.H. influence (Leaf, 1953; Tarail, 1953; Leaf, in preparation for publication). Variations in urine flow depend on changes both in A.D.H. activity and in the rate of urinary solute excretion (Talbot, 1952). Major changes in urine flow may be determined solely by alteration in solute output (Beaser, 1947; Brodsky, 1951; Talbot, 1952).

A more specific response index is suggested on consideration of the physiology of the antidiuresis referable to the posterior pituitary hormone. The primary influence of A.D.H. is to promote the reabsorption of water from the intraluminal renal tubular fluid as a final step in the elaboration of urine (Smith, 1951). This process takes place within certain osmotic limits (Talbot, 1952). Within this range, the action of A.D.H. should be directly reflected by the ratio of water to total osmotically effective solute in the urine. Since no other substance is known to have a direct action at the renal tubular epithelium comparable to that of A.D.H., change in "water concentration" of urine, this term being defined as the ratio of water to total osmotically effective solute, should prove to be a specific response index for use in A.D.H. assay.

The assay method to be reported here is a product of the reasoning outlined above. In addition, it was felt that a more specific assay might help to resolve some of the controversies concerning the role of A.D.H. in clinical disorders of water metabolism.

## MATERIALS AND METHODS

Male rats of the Sprague-Dawley strain averaging 250 gm. in weight have been employed. Before the animals are used for assay purposes they are trained to accept without struggle intragastric intubation with a number 8-F, soft rubber catheter and intraperitoneal injection of isotonic saline. Once satisfactorily trained the rats have been used repeatedly for assay purposes.

At a regular period each day the animals are fed a synthetic diet described in an earlier communication from this laboratory (Crawford, 1950). The intestinal residue of this fiber-free diet is so small that fecal contamination of urine collections is rarely encountered. The standard diet also insures a relatively constant solute output by the kidney.

Collections of spontaneously voided urine are made in individual metabolism cages. These are equipped with removable, stainless steel wire mesh floors to facilitate washing.

Six animals, last fed 16 to 18 hours earlier, are employed for each assay. The rats are hydrated at the start of the procedure by intragastric delivery of 350 ml. of water per square meter of body surface area at 37° C.[2] Diuresis begins about 45 minutes later. Seventy-five minutes after the initial hydration, the animals are again intubated and given 250 ml. of water per square meter. They are then placed in the individual metabolism cages for urine collections. A volume of at least 4 ml. of water-clear urine voided over the next 45-minute period suggests that an animal is behaving in a manner suitable

[2] Surface area is calculated on the basis of body weight according to the formula of Benedict (1932): S. A. (sq. m.) = $0.00092\sqrt[3]{(\text{weight in grams})^2}$.

for assay purposes. This preliminary collection is discarded after inspection and estimation of volume. Thereafter, urine is collected for 3 consecutive 45-minute periods. These specimens are labelled in order, $U_1$, $U_2$ and $U_3$. Intubation and delivery of 250 ml. of warm water per square meter of body surface area is repeated between $U_1$ and $U_2$ and again between $U_2$ and $U_3$. Between collection of $U_1$ and $U_2$ an intraperitoneal injection of 0.5 ml. of 0.87% NaCl solution is given. The material to be assayed is injected intraperitoneally in a volume of 0.5 ml. of isotonic saline between collection of $U_2$ and $U_3$.

The schedule of gastric intubation, and water administration has been designed to provide for a state of constant hypotonicity of body fluids and hypervolemia to inhibit release of endogenous A.D.H. (Verney, 1946; Welt, 1951; Straus, 1951; Leaf, 1952). Under experimental circumstances the procedure results in a sustained positive water balance equal to between 1.5 and 7% of the starting body weight of the animals. No evidence has been encountered which relates the sensitivity of response of the assay animal to the size of the water load within this range. Terminal, spontaneous voidings at the close of the first two 45-minute collection periods ($U_1$ and $U_2$) are readily obtained from the trained animals. The volumes of these will average approximately 5 ml. each. The volume of $U_3$ will be variable especially when the antidiuretic potency of the test material is great. When the A.D.H. content of the test material exceeds 0.5 milliunit, marked reduction of urine volume may make difficult collection of a representative, accurately timed urine sample. Under this circumstance the results of the test can be considered only of qualitative significance and a second assay using more dilute material should be performed.

The water concentration of the three sets of urine samples is estimated from determination of freezing point by a method previously described (Crawford, 1952).[3] The results are expressed in terms of the relation of water to solute in an ideal solution which freezes at the same temperature as the unknown and which has a molal freezing point depression of 1.860° C.

The data obtained from any individual animal are discarded from the series if either of the following types of behavior are observed: (a) If the first urine collection ($U_1$), shows a water concentration of less than 10 ml. per mosM; (b) if the urine collection immediately following the intraperitoneal injection of saline ($U_2$) shows a decrease in water concentration of 10% or more as compared with the concentration of the control specimen ($U_1$). Since both intubation and intraperitoneal injection of isotonic saline regularly result in a decrease in water concentration of the urine in untrained animals, presumably due to endogenous A.D.H. release (Rydin, 1938; O'Connor, 1942; Kelsall, 1949; Ames, 1953), these reactions suggest inadequate training of the assay animal. Responses are calculated from the remaining data as the per cent change in water concentration of $U_3$ as compared with $U_2$:

$$\text{Response} = \frac{\frac{\text{ml. } H_2O}{\text{mosM.}} U_3 - \frac{\text{ml. } H_2O}{\text{mosM.}} U_2}{\frac{\text{ml. } H_2O}{\text{mosM.}} U_2} \times 100.$$

The results are averaged and the mean is taken as the index of the antidiuretic potency of the injected material.

---

[3] Many of the determinations reported here were performed using a modified, Model B Osmometer manufactured by Fiske Associates, 44 Bromfield Street, Boston, Massachusetts.

TABLE 1. RESPONSES TO GRADED DOSES OF PITRESSIN

| Dose (milliunits) | 0.0 | 0.10 | 0.16 | 0.25 | 0.35 | 0.40 |
|---|---|---|---|---|---|---|
| Number of assays | 20 | 22 | 15 | 30 | 20 | 11 |
| Mean response* | −2.4 | −13.1 | −20.4 | −26.4 | −33.7 | −35.2 |
| Standard error$_\bar{x}$† | ±3.8 | ± 3.7 | ± 4.1 | ± 6.3 | ± 2.7 | ± 4.6 |

$$* \text{ Response} = \frac{\frac{\text{ml. } H_2O}{\text{mosM.}} U_3 - \frac{\text{ml. } H_2O}{\text{mosM.}} U_2}{\frac{\text{ml. } H_2O}{\text{mosM.}} U_2} \times 100.$$

† Standard Error of the mean response of 6 animals.

## RESULTS

Table 1 gives the mean responses and variability encountered in assays of Pitressin (Parke Davis Co., Lot #L-350-K), freshly diluted in isotonic saline, in doses ranging from 0.1 to 0.4 milliunit.[4] The average responses are shown graphically in the dose-response curve of Figure 1. The data yield a rectilinear relationship when dose and response are thus plotted on semilogarithmic coordinates.

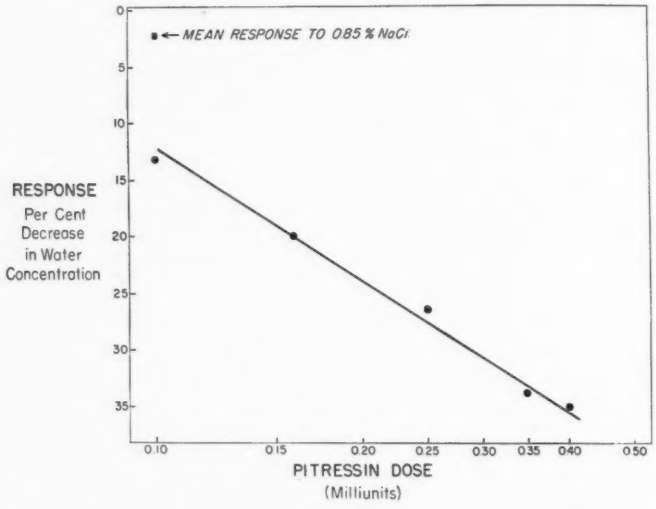


FIG. 1. Dose-Response Curve. The ordinate scale gives per cent decrease in urine water concentration. Pitressin dose is indicated on the abscissa. Mean responses to five dose levels of Pitressin are shown by the circle points. The average response to injections of 0.85% NaCl solutions containing no Pitressin is indicated by the square symbol.

TABLE 2. BLISS ANALYSIS OF ASSAY DATA FOR INDEX OF PRECISION (λ)

| | Responses to four doses | | | | $D_1=U_2-S_2$ | $D_2=U_1-S_1$ | $D_3=U_2-U_1$ | $D_4=S_2-S_1$ | $Y_1=D_1+D_2$ | $Y_2=D_3+D_4$ | $Y_3=D_1-D_1=D_1-D_2$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $U_2$ | $U_1$ | $S_2$ | $S_1$ | | | | | | | |
| 1 | −29 | −14 | −19 | −24 | −10 | +10 | −15 | + 5 | 0 | −10 | −20 |
| 2 | −26 | − 9 | −26 | −20 | 0 | +11 | −17 | − 6 | +11 | −23 | −11 |
| 3 | −17 | − 7 | −36 | −17 | +19 | +10 | −10 | −19 | +29 | −29 | + 9 |
| 4 | −18 | −15 | −38 | −34 | +20 | +19 | − 3 | − 4 | +39 | − 7 | + 1 |
| 5 | −24 | − 4 | −33 | −24 | + 9 | +20 | −20 | − 9 | +29 | −29 | −11 |
| 6 | −21 | − 8 | −34 | −34 | +13 | +26 | −13 | 0 | +39 | −13 | −13 |
| 7 | −17 | −21 | −50 | −21 | +33 | 0 | + 4 | −29 | +33 | −25 | +33 |
| 8 | −26 | −15 | −51 | −18 | +25 | + 3 | −11 | −33 | +28 | −44 | +22 |
| 9 | − 9 | −23 | −30 | −12 | +21 | −11 | +14 | −18 | +10 | − 4 | +32 |
| 10 | −19 | − 5 | −21 | −43 | + 2 | +38 | −14 | +22 | +40 | + 8 | −36 |
| | Doses $u_2=.16$ mU | | $s_2=.40$ mU | | | | | | $T_1=258$ | $T_2=-176$ | $T_3=+6$ |
| | $u_1=.10$ mU | | $s_1=.25$ mU | | | | | | | | |

I. $\lambda = S/b$;

$$S = \sqrt{\frac{\Sigma(Y^2) - \frac{(T_1^2+T_2^2+T_3^2)}{N}}{3N(N-1)}}$$

$b$ = The average slope of the dose response curve or

$$b = \frac{\Sigma D_3}{2N \log \frac{u_2}{u_1}} + \frac{\Sigma D_4}{2N \log \frac{s_2}{s_1}}$$

II. To derive value for $S$

1. $\Sigma(Y^2) = 18374$
2. $\frac{T_1^2+T_2^2+T_3^2}{10} = 9758$
3. $18374 - 9758 = 8616$
4. $\sqrt{\frac{8616}{30(9)}} = 5.7$

III. To derive value for $b$.

1. $\frac{\Sigma D_3}{20\left(\log \frac{.16}{.10}\right)} = -\frac{85}{20(.204)}$
2. $\frac{\Sigma D_4}{20\left(\log \frac{.40}{.25}\right)} = -\frac{91}{20(.204)}$
3. $\frac{-85}{4.08} + \frac{-91}{4.08} = -43.1$

IV. $\lambda = S/b$

$$\lambda = \frac{5.7}{-43.2} = -0.132$$

The index of precision (λ), has been calculated according to the method of Bliss (1944) for unselected results obtained from groups of 10 individual assay animals receiving respectively Pitressin doses of 0.10, 0.16, 0.25 and 0.40 milliunit. The value for this index which is, essentially, the square root of the ratio of error of variance to the slope of the dose-response curve permits comparison of the assay method reported here with that proposed by Stein (1952). The calculations are shown in Table 2.[5] The relatively small value for λ, 0.13, compares favorably with that calculated by Stein and his co-workers for their own method of assay (λ = 0.32) as well as for the methods of others which they reviewed.

## DISCUSSION

The method proposed here, though it might be referred to as "new," is basically one of the oldest techniques. Changes in urine specific gravity in response to water loading and deprivation are ordinarily interpreted to

[4] This material was assayed by the manufacturer using the standard procedure of the United States Pharmacopeia, XIV, and found to be 100% of label in September, 1951. No loss of activity during storage has been detected to date by the method of assay reported here.

[5] We are indebted to Mr. Roger Pinkham for his help in devising this table.

yield information concerning the responsiveness to A.D.H. of the kidney. However, changes in this measure of urine "water concentration" have also long been used to assay the capacity to produce A.D.H., particularly of individuals suspected of diabetes insipidus (Fussell, 1916). A technique quite comparable to that suggested here and including estimation by freezing point of water concentration in urine was employed ten years ago by Heller (1944) in his study of the capacity of newborn infants to secrete and to respond to A.D.H.

In an area so thoroughly explored as that of A.D.H. assay, the merits of a new method can be judged only after comparison with other methods of attributes such as economy, convenience, precision, sensitivity and specificity. In the first of these terms the procedure outlined here may be classed as relatively economical and convenient. The usefulness of the same assay animals on multiple occasions over periods of six or more weeks is a significant advantage.

It is generally true that the precision and sensitivity of biologic hormone assays will be enhanced if the test animal is rendered anatomically incapable of endogenous production of the hormone in question. The present assay method, like most of those which have been employed earlier for the same measurement, is open to the criticism that the subject animals have not been so prepared. In using this method in an early stage of its development Leaf and Mamby (1952) attempted to incorporate this refinement. Their results did not indicate that greater precision or sensitivity was thereby obtained (Leaf, personal communication). In the present procedure the training, sustained water loading and measurement of responses to control injections of inactive material appear to make of minor importance any loss of precision or sensitivity due to an endogenous secretory response on the part of the intact subject animals.

Comparison of the index of precision calculated on data obtained by the present method of assay (Table 2) with the values reported by Stein (1952) for his own method and others which he analyzed early suggested that this assay method offered significantly greater precision. Its sensitivity appeared to be at least equal to the better methods which had been proposed to that time. Very recently Dicker (1953) has reported yet another new method which, though it involves a complex and expensive apparatus, is clearly superior in terms both of precision and sensitivity to any method previously described. Unfortunately, Dicker's method where the index of response depends solely on change in urine volume may well prove lacking in specificity.

As has been pointed out earlier, changes in the rate of urine formation and in the rate of output of an individual solute or the sum total of urine solutes may occur in the absence of any alteration of A.D.H. influence at

the kidney (Beaser, 1947; Brodsky, 1951; Talbot, 1952; Leaf, 1953; Tarail, 1953; Leaf, in preparation for publication). In this connection, a consideration of the action of morphine is pertinent. This agent has been shown to exert a strong influence on diuresis (deBodo, 1944). In the intact, physiologically oliguric rat tested according to the Burn (1931) procedure, the injection of morphine causes a significant delay in excretion of the water load (Crawford, unpublished observations). In the intact, hydrated, polyuric rat prepared according to the Theobald (1934) procedure morphine administration is followed by sharp curtailment of the rate of urine flow (Crawford, unpublished observations). Thus using either of these two variants of the class of assays in which the response index is change in rate of urine flow, morphine is found to yield false positive, non-specific results. On the other hand, when the action of morphine on urinary chloride excretion is studied in an assay of the Ham (1943) type, a slight postadministration reduction in output is observed (Crawford, unpublished observations). Thus in an assay dependent upon change in rate of urine solute output, morphine or a substance with a similar action may interfere with measurement of A.D.H. The response index of the assay technique proposed here, is uninfluenced by the action of morphine (Crawford, unpublished observations).

While it is unlikely that morphine in quantities sufficient to cause interpretive difficulty would ever be contained in biologic fluids subject to assay, a review of these actions is useful to illustrate the lack of specificity in methods which depend simply on change in rate of urine flow or solute output. Of possibly greater physiologic importance is consideration of the influence on urine flow and composition of changes in glomerular filtration rate. There is high probability that changes in filtration do occur in assay animals. These may be in response to manipulations associated with water loading or intraperitoneal injections as well as consequent to the action of substances other than A.D.H. contained in the injected material (Smith, 1951). In polyuric dogs in which controlled changes in filtration rate were mechanically induced, Leaf has demonstrated correlated variations in urine flow and solute output although urine water concentration remained uninfluenced (Leaf, in preparation for publication). Hence, changes in filtration would be expected to interfere with A.D.H. assay procedures which depend on change in rate of urine flow or solute output but will be without influence on the response index of the method proposed here.

The assay method described has been used to date in a limited survey of A.D.H. titers of fresh antecubital venous blood serum. Sera have been assayed from normal human subjects moderately dehydrated by water deprivation and from edematous children with lipoid nephrosis. These two groups of subjects were chosen because others using assay methods depend-

ent on change in rate of urine flow have reported in such sera the presence of relatively high titers of A.D.H. (Stein, 1952; Galan, 1953). These preliminary explorations have failed to confirm the presence of comparably large amounts of hormone. It is believed that this discrepancy is referable to differences in specificity of the assay methods. Further explorations in these areas are being conducted.

## SUMMARY

A biologic method for the assay of antidiuretic hormone has been described which differs from the majority of those proposed heretofore in that the index of activity is taken as the change in urine water relative to total, osmotically effective solute. The assay offers a high degree of sensitivity and precision. The probability that this method may offer greater specificity for detection of antidiuretic hormone is discussed.

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# EFFECTS OF ALTERING THE BALANCE BETWEEN PROLACTIN AND OVARIAN HORMONES ON INITIATION OF LACTATION IN RABBITS[1]

JOSEPH MEITES AND JAMES T. SGOURIS

*Department of Physiology and Pharmacology, Michigan State College, East Lansing, Michigan*[2]

RECENTLY we reported (Meites and Sgouris, 1953) that the ability of a moderate dose of prolactin to initiate milk secretion in rabbits with well developed mammary glands, could be effectively inhibited if optimal, mammary growth-promoting doses of estrone and progesterone were injected at the same time. In the absence of either one or both of the steroid hormones, this dose of prolactin elicited a good degree of lactation. The present experiment was undertaken to determine the lactational response in rabbits to injections of (a) large doses of prolactin during treatment with a constant but optimal combination of estrone and progesterone for mammary development and (2) a moderate dose of prolactin in the presence of less than optimal amounts of the two steroid hormones.

## EXPERIMENTAL

Thirty-six New Zealand White rabbits, weighing 5 to 6 pounds each, were ovariectomized and permitted 10 to 14 days recovery to remove the influence of the ovarian hormones. Each rabbit was then injected subcutaneously with .096 mg. of estrone and 1 mg. of progesterone[3] daily in separate 0.1 cc. volumes of corn oil for 25 days. This combination of the two hormones was shown by Scharf and Lyons (1941) to elicit optimal mammary development in male rabbits, and was used by us in our previous experiments. From the 26th to 35th days, 3 groups of 6 rabbits each were continued on the same doses of the 2 steroid hormones, together with 2, 4 or 8 mg. of prolactin[4] daily, respectively; 3 other groups of 6 each were injected with 2 mg. of prolactin daily together with none,

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[3] The crystalline progesterone and estrone were furnished through the courtesy of Dr. J. O. Reed of Chas. Pfizer and Co., Inc.

[4] The purified prolactin was made available through the courtesy of E. R. Squibb and Sons, and was stated to contain 20–25 I.U. per mg. Prolactin was injected for 10 days in order to obtain a maximum lactational response with a minimum amount of prolactin. The quantity of milk found at the end of 10 days is considered to represent the total lactational response to prolactin.

¼ or ½ of the amounts of the 2 steroid hormones used previously. The prolactin was injected subcutaneously in 0.1 cc. volumes of distilled water. The rabbits were killed on the 36th day and their mammary glands were exposed and rated visually for lactational response, from 0 to 4. Commercial rabbit pellets and water were available at all times to the animals.

## RESULTS

The findings are summarized in Table 1. The 2 mg. daily dose of prolactin initiated an abundant lactation in the absence of the two steroid hormones (Group 1), while the same dose of prolactin was almost completely ineffective when injected together with .096 mg of estrone and 1 mg. of progesterone daily (Group 2). This is in agreement with our previous

TABLE 1. EFFECTS OF DIFFERENT LEVELS OF PROLACTIN, ESTRONE AND PROGESTERONE ON LACTATIONAL RESPONSE IN RABBITS

| Group | Daily treatment (26th–35th days of experiment) | Avg. lactational rating per gland |
|---|---|---|
| 1 | 2 mg. Prolactin | 4.0 |
| 2 | 2 mg. Prolactin +.096 mg. Est.* +1 mg. Prog.† | .2 |
| 3 | 4 mg. Prolactin +.096 mg. Est. +1 mg. Prog. | 1.9 |
| 4 | 8 mg. Prolactin +.096 mg. Est. +1 mg. Prog. | 3.2 |
| 5 | 2 mg. Prolactin +.048 mg. Est +.5 mg. Prog. | 1.1 |
| 6 | 2 mg. Prolactin +.024 mg. Est. +.25 mg. Prog. | 1.5 |

* Est. = estrone.
† Prog. = progesterone.

observations in rabbits under identical treatment. In the presence of the above amounts of the 2 steroids, 4 mg. of prolactin daily elicited a moderate lactational response (Group 3), while 8 mg. of prolactin daily (Group 4) produced almost as much milk as in group 1. When the 2 steroid hormones were injected in reduced amounts although in the same ratio as used previously (Groups 5 and 6), the 2 mg. daily dose of prolactin was sufficient to elicit at least a small amount of milk secretion. This was greater than in Group 2 but was considerably less than in Group 1.

## DISCUSSION

The present experiment is believed to demonstrate that (a) levels of estrogen and progesterone which are optimal or near optimal for mammary development in rabbits do not completely inhibit lactation if sufficiently large doses of prolactin are injected at the same time and (b) when the 2 steroid hormones are injected in less than optimal amounts for mammary growth, even a moderate dose of prolactin can elicit at least a small degree of milk secretion. This supplements our previous observations (Meites and Sgouris, 1953) and indicates that *the antagonism between mammary growth and lactation is relative, depending on the balance between the levels of prolactin and the two ovarian hormones in the body.* If the growth

stimulus exerted by the two ovarian hormones on the mammary glands is greater than the lactational stimulus of prolactin, then milk production will be inhibited, and *vice versa*. This does not exclude the possibility that other hormonal or non-hormonal factors may modify the lactational response.

Several previous reports which were not easily explainable at the time are in accord with the present observations. Although earlier workers had been unable to initiate lactation in pregnant animals with prolactin extracts without provoking abortion or resorption of the embryos (Nelson, 1936; DeFremery and Denekamp, 1935), Meites and Turner (1947) found that intraductal injections of 10 to 20 I.U. of prolactin into a single nipple of pregnant rabbits was able to induce a localized lactation under the injection site without terminating pregnancy. Apparently these amounts of prolactin were sufficient to overcome the inhibitory influence of the ovarian hormones on the local mammary area stimulated. Desclin (1952) has provided further evidence that large doses of prolactin can overcome the inhibitory action of the ovarian hormones on mammary secretion. He observed that when 30 I.U. of prolactin were injected daily into mature female rats, there was intense luetinization of the ovaries but no mammary secretion; however, when 200 I.U. of prolactin were injected daily, there was mammary secretion despite intense luteinization of the ovaries. Earlier, Nelson (1934) had reported that lactation in parturient guinea pigs was not suppressed by estrogens if a sufficient dose of prolactin was injected at the same time.

It is believed that the absence of milk flow during pregnancy or the presence of abundant lactation in animals which are simultaneously pregnant and lactating, can be accounted for on the basis of the balance present between the levels of prolactin and ovarian hormones in the body. During a non-lactational pregnancy, the combined action of estrogen and progesterone stimulates mammary development but renders it relatively refractory to prolactin stimulation. Thus mammary tissue from pregnant rats has much less capacity to respond to prolactin stimulation *in vitro* than mammary tissue from parturient rats (Folley, 1952; Sgouris and Meites, 1953). In addition, the secretion rate of prolactin by the pituitary during a non-lactational pregnancy is too low to initiate abundant lactation (Meites and Turner, 1948, a, b). Following parturition, prolactin secretion greatly increases and at the same time the reduction in ovarian hormones increases the sensitivity of the mammary glands to prolactin stimulation, serving to initiate copious milk flow. Lactation can continue in parturient animals which subsequently become pregnant, probably because (a) the initial level of prolactin secretion is already high and remains high as a result of the continued milking stimulus (Reece and Turner, 1937; Meites and Tur-

ner, 1942) and (b) estrogen and progesterone are secreted at lower levels during early than in late pregnancy, and hence they would not appreciably antagonize prolactin action and decrease milk production until the latter part of gestation. Thus, in dairy cows which are simultaneously lactating and pregnant, there is no pronounced drop in milk yield until after the 5th month of gestation, following which time the decline is about 20% greater than in non-pregnant cows (Hammond, 1927; Turner, 1939).

## SUMMARY

Thirty-six young rabbits were ovariectomized, and after 10 to 14 days were injected daily with 0.96 mg. of estrone and 1 mg. of progesterone for 25 days to induce optimal mammary development. From the 26th to 35th days, 3 groups of 6 rabbits each were continued on the same doses of steroid hormones together with 2, 4 and 8 mg. of prolactin daily, respectively; 3 other groups of 6 each were injected with 2 mg. of prolactin daily together with 0, $\frac{1}{4}$ or $\frac{1}{2}$ of the 2 steroid hormones used previously. On the 36th day the rabbits were killed and their mammary glands were exposed and rated visually for lactational response.

In the first 3 groups lactation was almost completely inhibited when only 2 mg. of prolactin was injected daily in the presence of the 2 steroid hormones, while 4 or 8 mg. daily elicited good lactational responses. In the second 3 groups, the greatest amount of milk was secreted when 2 mg. of prolactin was given daily without the steroid hormones, while the same dose of prolactin in the presence of either $\frac{1}{4}$ or $\frac{1}{2}$ of the steroid hormones produced a smaller flow of milk.

It is concluded that the antagonism between mammary growth and lactation is relative, depending on the balance between the levels of prolactin and the 2 ovarian hormones in the body. When the mammary growth stimulus exerted by the 2 ovarian hormones is greater than the lactational stimulus of prolactin, milk flow will be inhibited, and *vice versa*. The application of these findings to explain the absence of lactation during a non-lactational pregnancy or the simultaneous coexistence of lactation and pregnancy is discussed.

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# RADIO-IODINE INDUCED ATHYROID JOINT DISEASE IN MICE OF DIFFERENT STRAINS[1]

RUTH SILBERBERG AND MARTIN SILBERBERG

*Snodgras Laboratory, City Hospital Division, and Department of Pathology, Washington University, School of Medicine, Saint Louis, Missouri.*

RADIOTHYROIDECTOMY carried out after weaning inhibited the development of degenerative joint disease occurring in old mice. This effect was attributed to the retardation of growth and development of the articular cartilage caused by thyroid deficiency. This interpretation seemed to be correct, since radiothyroidectomy performed in adult mice of strain DBA did not prevent osteoarthritic lesions from appearing in old age. In addition, however, in DBA mice severe articular changes were observed which were quite characteristic and thought to be due to lack of thyroid hormone (Silberberg and Silberberg 1954). The aim of the present experiments was to extend the previous investigations to mice of a strain which differs biologically in many respects from strain DBA.

## MATERIAL AND METHODS

Fifty-five male mice of the closely inbred strain C57BL raised in our laboratory and kept on a stock diet of Purina Laboratory Chow and water *ad libitum* received 400 $\mu$c of radio-iodine, $I^{131}$, at the age of six months. A number of animals were killed at 12, 18 or 24 months; others were sacrificed as their conditions warranted it; still others died from hypophyseal tumors or from unrelated causes. At necropsy, a procedure was followed that has been described previously. The hypophyses were examined for the presence of lesions. From the neck region any residual or regenerated thyroid tissue found was taken out for microscopic study. The legs were removed, fixed, decalcified, sectioned in a sagittal direction, and stained with hematoxylin and eosin, or Periodic Acid Schiff reagent. This material was compared with that obtained from previous experiments and consisting of 49 male mice of strain DBA which also had received the isotope at the age of six months.

## OBSERVATIONS

*Thyroids:* The thyroids of mice of strain DBA are ordinarily more active than those of strain C57BL (Fig. 1 and 2).

Doses of 250–400 $\mu$c are adequate to effectively destroy the thyroid of

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mice. Grossly, the thyroid region of mice thus treated was transformed into a band of scarred tissue. Microscopically, the latter occasionally contained a few scanty atrophic and distorted acini embedded in fibrous tissue. While these acini may still be capable of taking up tracer doses of $I^{131}$, they do not undergo compensatory hypertrophy (Furth, Burnett and Gadsden 1953). Therefore a state of advanced, if not complete, thyroid deficiency exists in mice treated with adequate doses of $I^{131}$. Additional evidence for the existence of functional athyroidism is furnished by the occurrence of hypophyseal tumors which do not grow in the presence of thyroid hormone (Gorbman 1952).

*Hypophyses:* After it had been established that the hypophyseal lesions caused by injection of $I^{131}$ had the same structure as similar changes observed by other investigators (Gorbman 1949, Furth 1951), no further microscopic studies of these lesions were carried out. As was done previously (Silberberg and Silberberg 1954), the hypophyseal changes were classified according to their size into slight enlargement (+), and tumors +, the latter group comprising nodules larger than 2 mm in at least one diameter. The findings are presented in Table 1 together with those obtained in mice of strain DBA treated correspondingly.

TABLE 1. FINDINGS IN THE HYPOPHYSES OF MALE MICE OF STRAINS C57 AND DBA INJECTED WITH $I^{131}$ AT THE AGE OF SIX MONTHS

| Strain | Age group months | No. of mice | Mean age at death months | Mean post-radiation interval months | Findings in hypophyses* % | | |
|---|---|---|---|---|---|---|---|
| | | | | | 0 | (+) | + |
| C57 | 12 | 10 | 12.0 | 6.0 | 40.0 | 30.0 | 30.0 |
| DBA | 12 | 13 | 12.0 | 6.0 | 92.3 | 7.7 | 0.0 |
| C57 | 13–18 | 23 | 17.7 | 11.7 | 43.5 | 13.0 | 43.5 |
| DBA | 13–18 | 25 | 16.6 | 10.6 | 64.0 | 32.0 | 4.0 |
| C57 | 19–24 | 22 | 21.0 | 15.0 | 68.2 | 4.5 | 27.3 |
| DBA | 19–24 | 11 | 19.3 | 13.3 | 63.7 | 0.0 | 36.3 |
| All animals | | | | | | | |
| C57 | 12–24 | 55 | 17.8 | 11.8 | 50.9 | 14.5 | 34.6 |
| DBA | 12–24 | 49 | 16.0 | 10.0 | 71.4 | 18.4 | 10.2 |

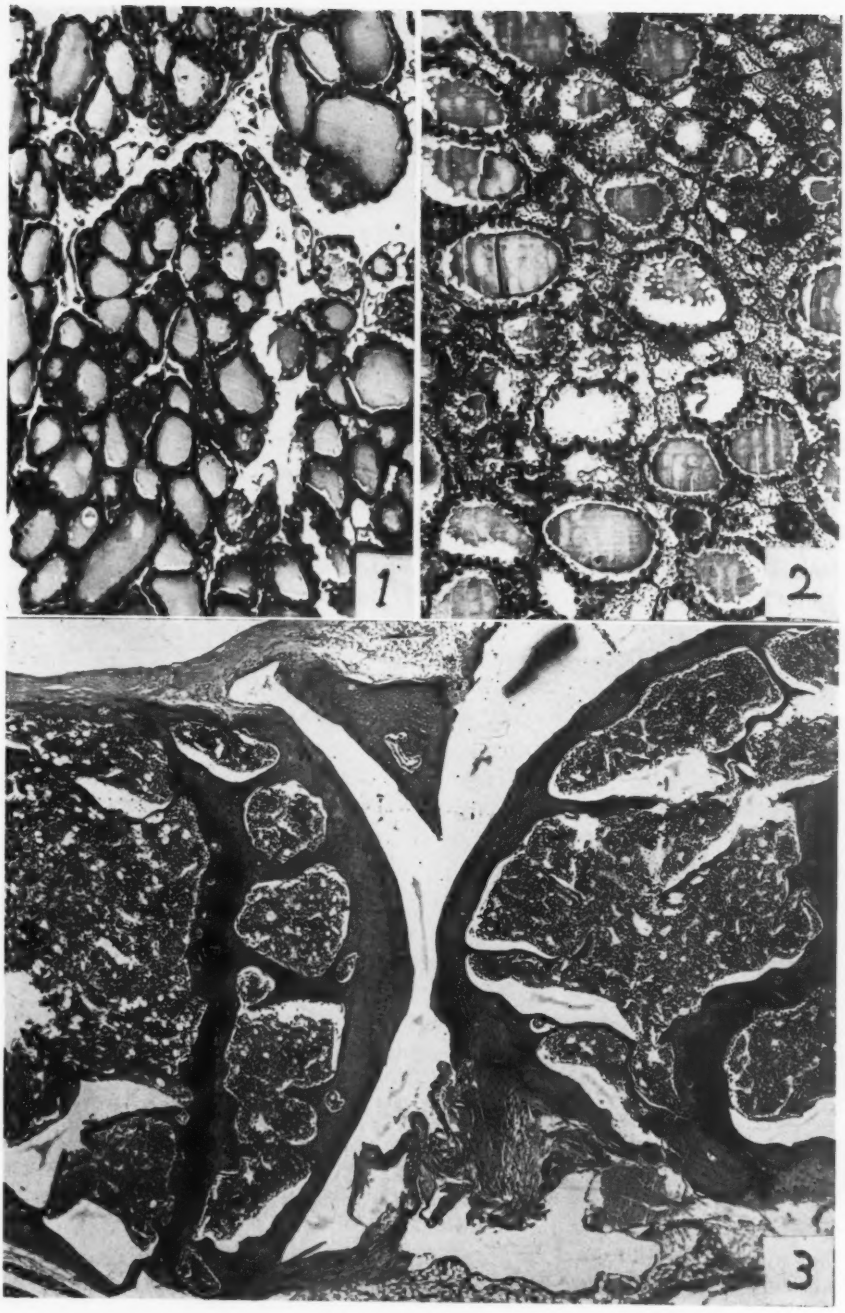
* (+) Slight enlargement.
\+ Tumors at least 2 mm. in one diameter.

(EXPLANATION OF FIGURES 1–3)

FIG. 1. Section of the thyroid of a C57 mouse. The acini are filled with hard colloid and lined by low epithelium. ×200.

FIG. 2. Section of the thyroid of a DBA mouse. The acini are large, lined by cuboidal or low columnar epithelium, the colloid is soft. ×200.

FIG. 3. Section through the kneejoint of a DBA mouse. Untreated control showing the usual architecture. ×40.




FIGS. 1–3

The total incidence of hypophyseal tumors was 34.6% in mice of strain C57BL; that of corresponding DBA mice was 10.2%. The hypophyseal neoplasms were not only more numerous but they appeared earlier in mice of strain C57BL than in DBA mice. Thus, 6 months after irradiation, 30% of the C57 mice had hypophyseal tumors, while as late as 10.6 months after injections of the isotope only 4% of the DBA mice had hypophyseal tumors. In the oldest age group of C57 mice, the tumor incidence dropped from the previous high—obviously because many animals with hypophyseal neoplasms had died at an earlier age—, while in DBA mice the tumor incidence only then reached its peak.

*Findings in the joints*

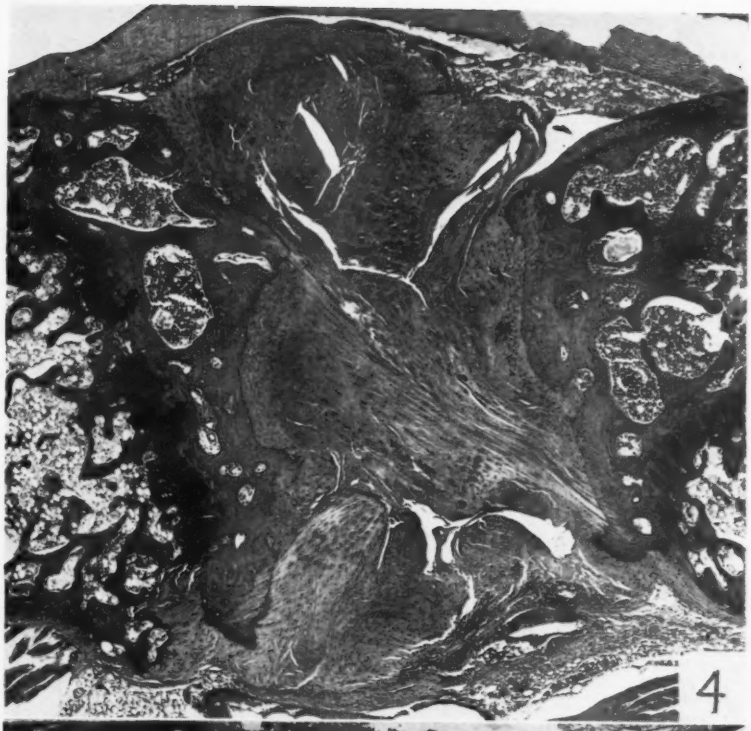
*Microscopic observations:* The changes occurring in the joints of athyroid mice of strain DBA have been fully described elsewhere (Silberberg and Silberberg 1954), and are together with a normal joint illustrated in Figures 3 and 4. Briefly, the lesions were degenerative in nature and involved primarily the menisci, ligaments and synovialis. The menisci failed to ossify, or if ossified at time of irradiation, they reverted to fibrohyaline tissue. The ligaments were edematous, and at late stages of the disease, thickened; the synovialis proliferated and became fibrosed. Ultimately, the entire epiphysis became flattened and distorted due to retarded growth and replacement of the bone marrow by fibrous tissue. The articular cartilage—in contrast to changes seen in osteoarthritis—responded only secondarily with growth or, in advanced cases, with degeneration.

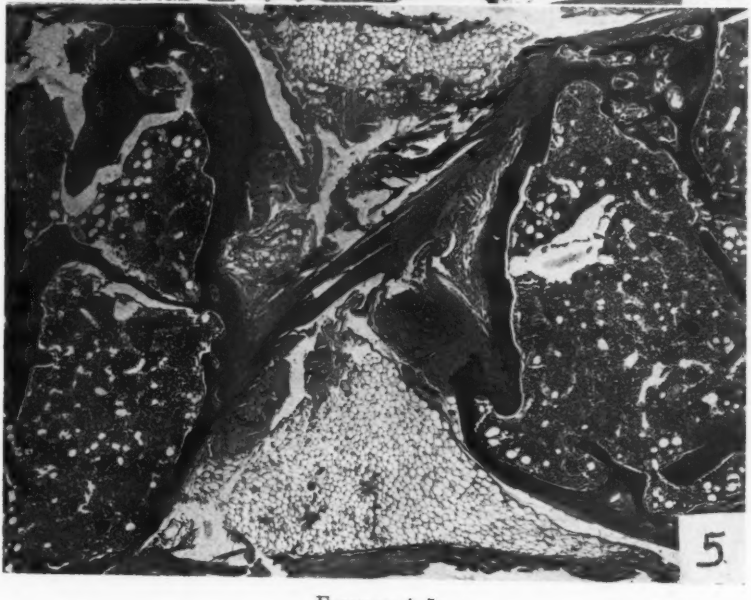
In the present series of male mice of strain C57BL, the athyroid arthropathy was neither as extensive nor as frequent as that observed in DBA mice treated correspondingly. Typical changes as occurring in C57 mice are demonstrated in Figure 5. *Slight changes:* The cartilage of the menisci was swollen or vacuolated but never underwent liquefaction as seen in mice of strain DBA. Mucoid and Schiff positive material appeared in increased amounts in the menisci, ligaments and synovialis; but again this increase was less conspicuous than in DBA mice. The ligaments were thickened, the cells swollen, and the synovialis proliferated. Occasionally

EXPLANATION OF FIGURES 4–5

FIG. 4. Section through the kneejoint of a DBA mouse injected with $I^{131}$. Moderately advanced arthropathy. The meniscus is enlarged and consists of fibro-hyaline tissue. The markedly thickened ligaments together with the enlarged meniscus obliterate the joint cavity. The epiphysis of the tibia is flattened. ×40.

FIG. 5. Section through the kneejoint of a C57 mouse injected with $I^{131}$. The changes are slight as compared with those shown in Fig. 4. The ligaments are slightly thickened and there is some proliferation of the synovialis in addition to some swelling and mucoid change. The dark areas in the ligaments will stain positive with PAS reagent. ×40.




FIGURES 4–5

TABLE 2. FINDINGS IN THE KNEEJOINTS OF MALE MICE OF STRAINS C57 AND DBA INJECTED WITH $I^{131}$ AT SIX MONTHS OF AGE

| Strain | Age group | No. of mice | Mean age at death (months) | Findings in joints* % | | |
|---|---|---|---|---|---|---|
| | | | | 0 | (+) | + |
| C57 | 12 | 10 | 12.0 | 90.0 | 10.0 | 0.0 |
| DBA | 12 | 13 | 12.0 | 92.3 | 0.0 | 7.7 |
| C57 | 13–18 | 23 | 17.7 | 52.1 | 47.9 | 0.0 |
| DBA | 13–18 | 25 | 16.6 | 36.0 | 24.0 | 40.0 |
| C57 | 19–24 | 22 | 21.0 | 59.1 | 27.3 | 13.6 |
| DBA | 19–24 | 11 | 19.3 | 36.4 | 27.2 | 36.4 |
| All animals | | | | | | |
| C57 | 12–24 | 55 | 17.8 | 61.8 | 32.7 | 5.5 |
| DBA | 12–24 | 49 | 16.0 | 51.0 | 18.4 | 30.6 |

* (+) Slight athyroid joint disease.
\+ Advanced athyroid joint disease.

*advanced lesions* were seen: The latter were characterized by penetration of the synovialis into the epiphyseal bone marrow and focal replacement of the latter by fibrous tissue. Further hyalinization of ligaments and synovialis led to some degree of obliteration of the joint cavity. The articular cartilage showed focal hyperplasia, hypertrophy, or degeneration.

*Incidence of athyroid joint disease:* In the following Table 2, the articular findings in male mice of strains C57BL and DBA given $I^{131}$ at six months of age are compiled. Slight changes are designated by the symbol (+), and advanced changes by + respectively.

As seen from the table, the incidence of articular lesions was lower in mice of strain C57BL than in those of strain DBA in spite of the fact that —at least in the two oldest age groups—the mean age at death was higher in C57 mice than in DBA mice. The total incidence of advanced athyroid joint disease was 5.5% and 30.6% for the two strains respectively. In mice of strain C57, the onset of the disease was also markedly delayed; at a mean age of 16.6 months 40% of the DBA mice had advanced arthropathy, while none of the C57 mice showed these lesions. In addition to the latter, a considerable number of mice showed articular changes typical of osteoarthritis namely hyperplasia, hypertrophy and degeneration of the articular cartilage associated with fraying and roughening of the surface of the joint. The incidence of these osteoarthritic lesions was about twice as high as in mice with intact thyroids of similar age.

## DISCUSSION

In male mice of strain C57BL treated with $I^{131}$ at the age of six months, the incidence of athyroid joint disease was about $\frac{1}{6}$ of that seen in male mice

of strain DBA treated correspondingly. Moreover, in mice of strain C57, the onset of the arthropathy was retarded and the severity of the lesions was strikingly decreased as compared to conditions in DBA mice. By contrast, mice of strain C57 developed more hypophyseal tumors than DBA mice. The discrepancies between the incidence of hypophyseal neoplasms on the one hand and that of articular lesions on the other indicate that secretions given off by the former do not play a major role in the pathogenesis of athyroid joint disease at least as far as adult mice and mice of strain C57BL are concerned.

The cause of the strain differences in skeletal susceptibility to athyroidism is not immediately clear. Different degrees of thyroid deficiency produced by $I^{131}$ cannot account for these results. As discussed in connection with the findings in the residual thyroid tissue, hypophyseal tumors do not grow in the presence of thyroid hormone (Gorbman 1952, Furth, Burnett and Gadsden 1953). These tumors were even more numerous in mice of strain C57BL than in DBA mice. Therefore, the thyroid deficiency produced by the isotope must have been at least as marked in mice of strain C57 as it was in DBA mice.

The difference in susceptibility to the arthropathy may, however, be related to differences in the physiological activity of the thyroids of the two strains (Barry and Kennaway 1933, Loeb and Kirtz 1939, Gruneberg 1943; 1947, Silberberg and Silberberg 1950). Since hyperthyroid individuals are more sensitive to elimination of thyroid hormone than individuals with less active thyroid (Means 1948, Hurxthal and Musulin 1953), thyriod deficiency may have more conspicuous manifestations in mice belonging to the relatively hyperthyroid strain DBA than in the relatively hypothyroid C57 mice.

The strain differences in the incidence of hypophyseal neoplasms induced by $I^{131}$ may also be related to variations in thyroid activity of the respective strains. The less active thyroids of C57 mice might concentrate less of the isotope than the more active thyroids of DBA mice. This in turn would in mice of strain C57 allow more radioactive material to reach the hypophyses than in mice of strain DBA. The hypophyses of the former would thus be exposed to larger amounts of radiation and respond with more tumor growth than the hypophyses of the latter.

The occurrence of osteoarthritis in C57 mice radiothyroidectomized after cessation of growth and the rise in the incidence of these lesions above that seen in untreated mice confirm our earlier observations in mice of strain DBA. Apparently, the inhibition of osteoarthritic lesions in mice radiothyroidectomized during growth is due to incomplete development of the articular cartilage. This problem will be dealt with at a later date.

SUMMARY

Male mice of strain C57BL radiothyroidectomized with $I^{131}$ at the age of six months develop a characteristic joint disease as did male mice of strain DBA treated similarly. In C57 mice, however, this athyroid arthropathy is much lesss frequent and strikingly less severe than in DBA mice. These strain differences in susceptibility to athyroidism are tentatively explained on the basis of strain differences in thyroid activity in animals of the two strains, DBA mice showing physiologically hyperactive thyroids as compared to those of C57 mice. In regard to $I^{131}$-induced hypophyseal neoplasms, the susceptibility of the two strains was reversed: C57 mice had a higher incidence of these tumors than mice of strain DBA.

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# MAINTENANCE OF PREGNANCY IN THE ABSENCE OF DIETARY PROTEIN WITH ESTRONE AND PROGESTERONE[1]

MARJORIE M. NELSON AND HERBERT M. EVANS
*Institute of Experimental Biology, University of California, Berkeley, California*

NELSON and Evans (1953a) have recently shown that institution of a protein-free diet the day of breeding in normal 80-day old rats resulted in 90–100% resorptions, with fetal death occurring in the majority of rats on the 9th or 10th days of gestation. The normality of reproductive performance in pair-fed controls receiving a 30% protein diet eliminated the factors of inanition or other dietary deficiencies as causes for these reproductive disturbances. The early appearance of vaginal erythrocytes and the rapidity of the resorptive process in these deficient rats together with the early loss of the estrous cycle in unbred rats suggested the possibility of hormonal deficiencies in these animals. Lyons (1943) has shown that the daily injection of 1 μg. estrone together with 3–4 mg. progesterone will maintain pregnancy in hypophysectomized-oophorectomized rats. The same dosages of estrone and progesterone will also maintain pregnancy in vitamin B6-deficient rats (Nelson, Lyons and Evans, 1951). Therefore, it was thought desirable to test the efficacy of these hormones, both singly and combined, in rats maintained on a protein-free diet.

## EXPERIMENTAL METHODS

Female stock rats of the Long-Evans strain, 75–85 days of age and averaging 190 gm. in body weight, were bred with normal males and placed on the protein-free diet the day of breeding, i.e. the day of finding sperm in the vaginal smear. All rats were weighed at regular intervals. Vaginal smears were examined daily during gestation for the presence of erythrocytes, the sign that implantation has occurred; the appearance of vaginal erythrocytes before the 12th day in the Long-Evans rat also indicates the presence of at least one resorbing site, i.e. fetal death and beginning resorption of one or more implantation sites (Nelson and Evans, 1951).

Each experimental group consisted of 10 to 14 animals. Two or three experimental groups were used for each hormone dosage, one group being autopsied on day 13 of gestation and the other groups on days 21–23 of gestation. At autopsy all fetuses were

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carefully examined and the implantation sites were measured in the antero-posterior diameter.

All injections were started 3 days after breeding. The hormones, estrone and progesterone, were dissolved in 0.1–0.3 cc. sesame oil and injected subcutaneously. In the first experiment daily dosage levels of 0.5 μg. estrone[2] and 4 mg. progesterone were given singly or combined. In the second experiment dosage levels of estrone alone varying from 1 μg. to 6 μg. were given daily. Two experimental groups received 0.3 cc. sesame oil daily and two groups were uninjected.

The protein-free diet (Nelson and Evans, 1953a), consisted of 88% sucrose, 8% hydrogenated cottonseed oil (Crisco or Primex) and, 4% salts.[3] Crystalline vitamins per kilogram of diet were: 50 μg. vitamin $B_{12}$, 600 μg. d-biotin, 10 mg. 2-methyl-1, 4-naphthoquinone, 10 mg. thiamine HCl, 10 mg. pyridoxine HCl, 11 mg. pteroylglutamic acid, 20 mg. riboflavin, 2 mg. p-aminobenzoic acid, 50 mg. niacin, 100 mg. d-calcium pantothenate, 800 mg. inositol, and 1.0 gm. choline chloride. All rats received weekly a fat-soluble vitamin mixture containing 800 U.S.P. units vitamin A, 115 chick units vitamin D, 6 mg. dl-alpha-tocopherol, and 650 mg. corn oil (*Mazola*).

## RESULTS

Table 1 summarizes the data from the first experiment. The protein-deficient animals not receiving hormones, showed 90–100% resorptions with early appearance of erythrocytes in the vaginal smear, days 9–10, due to early resorbing sites. When the animals were autopsied on day 13, implantation sites were observed in all animals, the average number per rat being 9.4 for the uninjected group and 9.3 for the group receiving sesame oil. These sites averaged 0.2–0.3 cm. in diameter in contrast with normal values of 1.0–1.1 cm. When the autopsies were delayed to the end of gestation, days 21–23, the implantation sites were extremely minute and could be found in only 60% of the animals, thus decreasing the average number per rat to 6.8 and 4.7, respectively.

The daily injection of *0.5 μg. estrone* was somewhat beneficial in that living young were found in 20–30% of the animals. Implantation sites were slightly larger at day 13 and at day 21 than the sites observed in the uninjected animals or those receiving sesame oil, thus indicating a later time of fetal death for animals injected with estrone. The majority of rats that resorbed exhibited from 1 to 5 days of cornified vaginal smears late in gestation, usually 15–18 days after breeding, thus indicating necrosis of all placental tissue with complete absence of placental gonadotrophic hormones. It may be noted that the appearance of vaginal erythrocytes occurred on days 10–11, slightly later than for the groups not receiving any hormones

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[2] Dosage levels of either 0.5 μg. or 1 μg. estrone have been used in combination with progesterone or luteotrophic hormone for both the hypophysectomized-oophorectomized rat (e.g. Averill, Ray and Lyons, 1950) and the $B_6$-deficient rat (Nelson, Lyons, and Evans, 1953).

[3] Salts no. 4 of Hegsted *et al.* (1941).

TABLE 1. MAINTENANCE OF PREGNANCY ON PROTEIN-FREE DIET WITH ESTRONE AND PROGESTERONE

| Experimental group[1] | Rats bred | Autopsy day of gestation | Wt. change during gestation | Onset vaginal RBC | Implantations | Resorptions of implants | Litters[2] of implants | Impl. sites avg. per rat | Living young per litter[3] |
|---|---|---|---|---|---|---|---|---|---|
| | No. | day | gm. | day | % | % | % | No. | No. |
| Uninjected | 10 | 13 | −28 | 9.8 | 90 | 89 | 11 | 9.4 | |
| | 10* | 22–23 | −41 | 10.6 | 90 | 89 | 11 | 6.8 | |
| Sesame Oil | 10 | 13 | −31 | 10.2 | 100 | 90 | 10 | 9.3 | |
| | 10 | 21 | −41 | 9.9 | 100 | 100 | 0 | 4.7 | |
| Estrone 0.5 μg. | 10 | 13 | −31 | 10.7 | 90 | 78 | 22 | 9.9 | |
| | 10 | 21 | −33 | 11.1 | 100 | 70 | 30 | 7.4 | |
| Progesterone 4 mg. | 10 | 13 | −30 | 13.0 | 100 | 20 | 80 | 8.7 | 5.9 |
| | 10 | 21 | −30 | 13.0 | 100 | 30 | 70 | 8.9 | 6.3 |
| | 10 | 21 | −32 | 13.3 | 100 | 30 | 70 | 8.8 | 6.1 |
| 0.5 μg. Estrone +4 mg. Progesterone | 10 | 13 | −28 | 11.8 | 100 | 0 | 100 | 9.7 | 8.4 |
| | 10 | 21 | −18 | 12.8 | 100 | 0 | 100 | 9.6 | 8.3 |
| | 10* | 22–23 | −22 | 12.9 | 100 | 0 | 100 | 8.6 | 7.4 |

[1] Injections were given daily, beginning 3 days after breeding. Animals were autopsied 13 days or 21–23 days after breeding as indicated. Food intake was measured in the two starred groups.

[2] This column gives the percentage of rats with living embryos at autopsy and does not imply that all rats littered, inasmuch as the day of autopsy varied from 13 to 23 days after breeding.

[3] This column gives the number of living embryos per litter, i.e. the number of normal implantation sites in rats with maintenance of pregnancy. Values are given only for groups in which at least 4 rats maintained pregnancy.

The daily injection of *4 mg. progesterone* was markedly beneficial as pregnancy was maintained in 70–80% of the animals. Three groups of animals were used in testing the effects of this hormone. Vaginal erythrocytes appeared normally on day 13 and the number of implantation sites per rat was also normal (9–10) for the 3 groups. The average number of living young per litter was, however, only 6 in comparison with the normal average of 8–9 young.

The combination of *estrone and progesterone* in the same dosage levels maintained pregnancy in 100% of the animals, three groups being used to confirm this effect. The number of living young per litter was close to normal values, averaging 7.4, 8.3 and 8.4 young for the three groups.[4] Food intake was measured in one group of rats which received estrone and progesterone and were autopsied on days 22–23; these rats were matched in body

[4] These values are only slightly below the average value of 9.1 young obtained for five groups of control animals (total 50 rats) of the same age receiving purified diets containing 15 to 25% protein; the group averages were 8.5, 9.0, 9.1, 9.4 and 9.4 young, respectively.

weight with the uninjected group autopsied at the same time. The group receiving estrone and progesterone, in which living young were maintained in all animals, averaged 8.2 gm. of food daily in contrast with a daily average of 9.2 gm. for the uninjected group, in which 90% of the animals resorbed.

For all groups the close correspondence in reproductive performance for animals autopsied on day 13 and for those autopsied on days 21–23 should be noted. All groups showed an average loss of 30–40 gm. in body weight per rat during gestation, regardless of the day of autopsy or the outcome of gestation, except the groups receiving the combination of estrone and progesterone. These groups showed an average loss in body weight of only 20 gm. per rat, presumably due to retention of water as these animals exhibited marked edema of the abdominal organs, especially the pancreas.

The maintenance of pregnancy in 20–30% of the animals injected with estrone alone, 0.5 μg. daily dosage, prompted a second experiment to study the effects of higher levels of estrone. Table 2 summarizes the data for animals receiving 1–6 μg. estrone daily and includes the groups receiving the 0.5 μg. level, those receiving sesame oil, and those uninjected.

The daily injection of 1 μg. estrone maintained pregnancy in 50% of the animals autopsied on day 13 and in 70% of those autopsied on day 21, an

TABLE 2. EFFECTS OF VARYING LEVELS OF ESTRONE ON REPRODUCTION OF RATS MAINTAINED ON A PROTEIN-FREE DIET

| Estrone dosage | Rats bred | Autopsy day of gestation | Wt. change during gestation | Onset vaginal RBC | Implantations | Resorptions of implants | Litters of implants | Impl. sites avg. per rat | Living young per litter |
|---|---|---|---|---|---|---|---|---|---|
| μg. daily | No. | day | gm. | day | % | % | % | No. | No. |
| 0 | 20 | 13 | −30 | 10.0 | 95 | 89 | 11 | 9.4 | |
| | 20 | 21–23 | −41 | 10.2 | 95 | 95 | 5 | 5.7 | |
| 0.5 | 10 | 13 | −31 | 10.7 | 90 | 78 | 22 | 9.9 | |
| | 10 | 21 | −33 | 11.1 | 100 | 70 | 30 | 7.4 | |
| 1.0 | 12 | 13 | −29 | 10.8 | 100 | 50 | 50 | 10.3 | 8.3 |
| | 10 | 21 | −30 | 12.7 | 100 | 30 | 70 | 9.5 | 9.0 |
| 2.0 | 10 | 13 | −28 | 12.0 | 100 | 20 | 80 | 9.6 | 7.4 |
| | 10 | 21 | −27 | 12.1 | 100 | 20 | 80 | 7.8 | 7.0 |
| 3.0 | 12 | 13 | −31 | 11.9 | 100 | 17 | 83 | 10.5 | 6.7 |
| | 14 | 21 | −31 | 10.6 | 100 | 36 | 64 | 8.8 | 6.4 |
| 4.0 | 12 | 13 | −32 | 10.8 | 100 | 50 | 50 | 10.8 | 4.8 |
| | 11 | 21 | −42 | 11.0 | 100 | 64 | 36 | 9.6 | 5.0 |
| 5.0 | 12 | 13 | −31 | 10.5 | 100 | 67 | 33 | 9.4 | 2.0 |
| | 11 | 21 | −37 | 10.6 | 100 | 64 | 36 | 9.4 | 3.0 |
| 6.0 | 13 | 13 | −28 | 9.3 | 100 | 92 | 8 | 10.2 | |
| | 11 | 21 | −42 | 10.7 | 100 | 82 | 18 | 9.8 | |

average of 60% for the combined groups. Increasing the dosage to 2 μg. daily resulted in maintenance of living young in 80% of the animals in both groups. Similarly a daily dosage of 3 μg. resulted in maintenance of pregnancy in 64–83% (average 73%) of the animals. Higher levels of estrone, 4 μg. and 5μg. were not as beneficial resulting in living young in 36–50% (average 43%) and in 33–36% (average 34%) of the animals in the respective groups. The highest level tested, 6 μg. daily, did not maintain pregnancy in the protein-deficient animals. The average number of living young per litter was normal (8–9 young) for the groups receiving 1 μg. daily but decreased as the estrone level was raised. The close correspondence in reproductive performance for animals autopsied on day 13 with those autopsied on day 21 was again observed.

The onset of vaginal erythrocytes varied directly with reproductive success in these groups, occurring closer to the normal time (days 12–13) in the groups where pregnancy was maintained. Cornified vaginal smears for 5–6 days, starting with day 15 or 16, were noted in all rats that resorbed while receiving 1 μg estrone daily. With higher estrone levels, 3–5 μg., only a few rats that resorbed exhibited cornified vaginal smears and these occurred later on days 19–20. With the highest estrone level, 6 μg., cornified vaginal smears were observed as early as day 10.

## DISCUSSION

The data presented in this study demonstrate that pregnancy can be maintained in all animals on a protein-free diet by injections of estrone and progesterone. Furthermore, the injection of either hormone separately can maintain pregnancy in the majority of the animals. The hormonal inadequacies of the protein-deficient rats are thus clearly shown. The deficiency of progestin may be due to (1) inadequate production or secretion of the pituitary luteotrophic hormone (lactogenic or mammotrophic hormone), (2) inadequate ovarian production or secretion of progestin in response to pituitary and placental stimulation, or (3) inadequate production or secretion of placental luteotrophin. Similarly, the deficiency of estrin may be due to (1) inadequate production or secretion of FSH and ICSH (LH) by the pituitary, (2) inadequate ovarian production or secretion of estrin in response to pituitary and placental stimulation, or (3) inadequate production or secretion of placental gonadotrophins which furnish estrin or stimulate estrin production (Averill, Ray and Lyons, 1950). Studies on these problems are in progress.

The maintenance of pregnancy in the majority of protein-deficient animals by moderate levels of estrone (1–3 μg.) may probably be interpreted as an indirect effect of estrone in stimulating the pituitary production and secretion of luteotrophin (e.g. Lyons, 1951; Nelson, 1951) which, in turn,

stimulates the ovarian production of progestin. In the animals receiving higher levels of estrone (4–6 μg.) apparently insufficient progestin is produced to balance the estrin available to the animal. These groups are similar to the B6-deficient animals which received 4 mg progesterone combined with 2, 3 or 6 μg. estrone (Nelson, Lyons and Evans, 1951) in which maintenance of pregnancy decreased as the estrone level increased. The presence of some circulating progestin would account for the failure of resorbing rats injected with high levels of estrone to exhibit cornified vaginal smears after fetal and placental death.

Measurement of food intake in protein-deficient animals has demonstrated that the maintenance of pregnancy by the combination of estrone and progesterone is not due to an increased food intake but, on the contrary, is associated with a slight decrease in food intake.

These protein-deficient rats are similar to the $B_6$-deficient rats previously studied and to the hypophysectomized-oophorectomized rat in that pregnancy can be maintained by the combination of estrone and progesterone. The number of living young per litter is, however, closer to normal values for the protein-deficient animals than for $B_6$-deficient or hypophysectomized-oophorectomized animals when pregnancy is maintained by the hormones. The maintanance of pregnancy in protein-deficient rats by progesterone alone reveals that the hormonal inadequacies of these animals are much less severe than those of either the $B_6$-deficient or the hypophysectomized-oophorectomized rat in which the combination of hormones is required. Furthermore, the maintenance of pregnancy by estrone alone in the protein-deficient rat shows a response, whether by the pituitary or by the ovary, that the $B_6$-deficient rat does not seem capable of making.[5]

SUMMARY

In the absence of dietary protein throughout the gestation period pregnancy was maintained in all animals injected with both estrone and progesterone and in the majority of the animals injected with either hormone alone. Reproductive success with estrone injections varied in accordance with the dosage used. Maintenance of pregnancy by the combination of hormones was not due to an increased food intake.

*Acknowledgments*

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[5] It should be mentioned that levels of estrone alone higher than 1 μg. daily were not tested in $B_6$-deficient animals.

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# THE PRODUCTION OF HYPERTENSION, NEPHROSCLEROSIS AND CARDIAC LESIONS BY METHYLANDROSTENEDIOL TREATMENT IN THE RAT[1]

ERNESTO SALGADO AND HANS SELYE

*The Institute of Experimental Medicine and Surgery, Université de Montréal, Montreal, Canada*

EARLIER investigations had shown that chronic overdosage with mineralo-corticoids, such as desoxycorticosterone acetate (DCA), can produce a syndrome of "hyalinosis." Its manifestations differ somewhat in various species. In the rat, the animal most commonly used for this kind of study, hyalinosis is characterized by nephrosclerosis (glomerular and arteriolar hyalinization, hyalin casts), a "rheumatic-like" myocarditis (partially hyalinized granulomatous nodules in the heart) and periarteritis nodosa (with hyalinization and inflammation, especially in the mesenteric and cerebral vessels). These morphologic changes are accompanied by hypertension and polyuria. Although, with prolonged and heavy DCA overdosage, this syndrome can be elicited even in the intact rat on a normal diet, its development is greatly accelerated by the "conditioning" effect of unilateral nephrectomy and a high NaCl intake. The hypothesis has been advanced that excess production of, or unusual sensitization to, endogenous mineralo-corticoids may be an important factor in the pathogenesis of comparable disease manifestations in man (Selye, 1950, 1952).

It has been objected against this interpretation that the changes produced by DCA may not be specific, they could be manifestations of an acquired hypersensitivity to this synthetic steroid. We considered this possibility to be somewhat far-fetched, since among several hundred related steroids, only those with mineralo-corticoid activity produced the hyalinosis syndrome, and it was thought unlikely that the body would develop hypersensitivity solely to this class of hormones (for literature on these divergent interpretations see: Selye, 1951; Selye and Horava, 1952, 1953). Besides, virtually identical changes have also been produced with anterior pituitary extracts, which caused adrenocortical enlargement. Here again unilateral nephrectomy and sodium had a sensitizing effect, while adrenalectomy prevented the hyalinosis-producing action of these hypophyseal

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preparations (Selye, 1951). Since adrenalectomy notoriously increases susceptibility to various hypersensitivity reactions, we thought it improbable that the pituitary extracts could have acted through a mechanism of allergic sensitization. It was assumed that they produced hyalinosis by virtue of some hormone (probably somatotrophin), whose efficacy in this respect, might be conditional upon endogenous mineralo-corticoids.

Recently, our views on the role of corticoids in the pathogenesis of hyalinosis and hypertension, met with an apparently much more serious obstacle. It was noted that a synthetic androgen, methylandrostenediol (MAD), can also produce essentially similar changes (Skelton and Grant, 1952). This compound is devoid of corticoid activity as judged by life-maintenance tests in adrenalectomized rats (Guillemin and Conti, 1953). It was quite justified to conclude therefore "that the production of similar lesions (hyalinosis) by an androgenic substance casts some doubt on the validity of such a concept" as that which ascribes them specifically to mineralo-corticoids (Skelton, 1953).

The possibility still remained, however, that MAD might not cause hyalinosis in itself but, rather, through the intermediary of some action upon the adrenal. This steroid is singularly effective in producing a storage, within adreno-cortical cells, of so-called "colloid granules", which exhibit a high affinity for eosin and fuchsin. Similar granules do occur on occasion in human adrenals (Vélican, 1948), and they are claimed to be particularly frequent in patients who died from hypertension and cardiovascular disease (Liebegott, 1944). It is noteworthy that experimentally, such colloid granules had first been produced in the adrenals of rats treated either with methyltestosterone (a compound closely related to MAD), or with DCA, in combination with the same anterior pituitary extracts which caused hypertension and hyalinosis (Selye and Stone, 1950). Significantly, these singular granules also occur in rats in which hyalinosis and hypertension are induced by the "endocrine kidney" technique (Pellegrini, 1951; Robert, 1953), or by other surgical procedures conducive to a primarily renal hypertensive disease (Rather, 1951).

These considerations led us to undertake experiments designed to explore the possible role of the adrenals in the production of hypertension and hyalinosis by MAD.

## MATERIALS AND METHODS

Fifty female Sprague-Dawley rats were subdivided into 4 groups, as outlined in Table 1. The right kidney was removed in all groups, and bilateral adrenalectomy was performed in Groups III and IV, at the beginning of the experiment.

Ten mg. of MAD[2] (17$\alpha$-methyl-$\Delta$5-androstene-3 $\beta$, 17 $\beta$-diol) microcrystals were ad-

[2] We are indebted to Mr. Roger Larose of the Ciba Company Limited, Montreal who supplied us with generous amounts of MAD for this work.

ministered daily, subcutaneously in 0.2 ml. of water, Tween 80 being used as a suspending agent.

Blood pressure measurements were made at frequent intervals by the tail-cuff method of Friedman and Freed (1949).

All animals were killed after 46 days of treatment and their organs were fixed in Bouin's solution, for weighing and histologic study. Sections were stained with hematoxylin-eosin, as well as with the PAS procedure (McManus, 1948).

## RESULTS

In the course of the experiment, it soon became evident that the *blood-pressure* and the *fluid-intake* (as well as the diuresis) rose significantly above normal only in the MAD-treated intact rats. The normal growth-rate, as reflected by *body-weight*, was not significantly retarded by adrenalectomy in these salt-treated animals, even when they were given MAD. Conversely, the intact animals treated with MAD, actually lost weight during the last ten days of observation and these—unlike those of the other groups—were manifestly ill as judged by their clinical appearance (cf. Figure 1). Two animals in this group died prior to the termination of the experiment, with manifest signs of nephrosclerosis, periarteritis and myocarditis. We also lost two animals in Group III and four in Group IV, but these apparently from adrenal insufficiency, since none of them showed any detectable manifestations of hyalinosis. These prematurely lost animals were excluded from the means of Table 1.

At autospy *nephrosclerosis, myocarditis, encephalopathy* (due to periarteritis nodosa of the cerebral vessels) and *mesenteric periarteritis* were detectable only in the intact-MAD-treated animals of Group II. An attempt was made to evaluate this change on a semi-quantitative basis, using a scale of 0 to +++. The percentage of positives in any one group was designated as "% incidence" and the means of the values obtained by grading were expressed as a percentage of the possible maximum, and designated as "%

TABLE 1. ORGAN WEIGHT IN SALT TREATED AND UNILATERALLY NEPHRECTOMIZED RATS TREATED WITH LARGE DOSES OF MAD

| Group and treatment | No. of rats | Organ weights in mg./100 gm. body weight | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Kidney | Heart | Adrenals | Spleen | Thymus | Preputial glands | Hypophysis |
| I Control | 8 | 760±26 | 351±12 | 25±1.1 | 265±25.3 | 157±7 | 40± 2.2 | 4.6±0.10 |
| II MAD | 13 | 1337±40 | 486±11 | 25±1.5 | 332±25.2 | * | 154±13.2 | 3.5±0.18 |
| III Adr-x | 10 | 659±27 | 322±16 | — | 245±11 | 210±3.5 | 51± 5.5 | 5.2±0.34 |
| IV Adr-x+MAD | 19 | 945±26 | 346±41 | — | 246± 6.2 | * | 127±10 | 4.0±0.13 |

All weights are accomplished by the standard error of the mean.
* Complete involution.

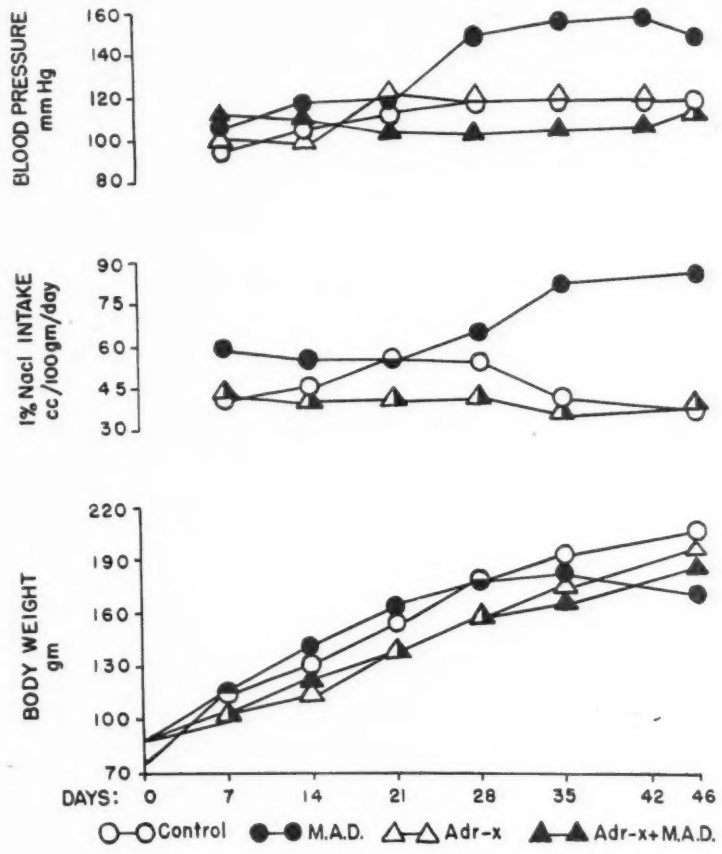


FIG. 1. Graphic representation of the changes in body-weight, fluid-intake and blood-pressure of normal and adrenalectomized, unilaterally nephrectomized rats, treated with high doses of MAD.

severity". The following is a summary of our findings in the MAD-treated-intact animals of Group II:

| | *% Incidence* | *% Severity* |
|---|---|---|
| Nephrosclerosis | 66 | 50 |
| Myocarditis | 56 | 55 |
| Encephalopathy | 15 | 19 |
| Mesenteric periarteritis[3] | 62 | 29 |

[3] While nephrosclerosis, myocarditis and encephalopathy were macroscopically evident at autopsy, the mesenteric periarteritis was clearly detectable only upon histologic examination in all but two rats of this group.

It is particularly noteworthy that all MAD-treated-intact rats had some manifestations of hyalinosis, at least in one of their organs, while we found no pathologic changes of this kind in any among the MAD-treated-adrenalectomized animals. In other words, ablation of the suprarenals, which notoriously sensitizes the organism to most damaging agents, actually offers complete protection against these manifestations of MAD-intoxication.

The means of the organ-weights are listed, together with their standard errors, in Table 1.

It will be noted that the mean *kidney* weight was greatest in the MAD-treated-intact rats (Group II), presumably because here, the nephrosclerotic action of this steroid was superimposed upon its renotrophic effect. However, the kidneys in the adrenalectomized-MAD-treated animals were also significantly enlarged, in comparison with the adrenalectomized controls ("P" $<0.01$). This was exclusively due to the renotrophic action of the steroid, since histologic examination revealed no trace of nephrosclerosis in Group IV.

The *heart* weight was significantly above normal only in the intact-MAD-treated animals. This is presumably an expression of the hypertension.

We attach particular importance to the fact that the *adrenals* were not enlarged by the MAD treatment, although their histologic structure was highly abnormal. This emphasizes the impossibility of drawing inferences as to function from the gross weight of these glands. It is evident that, all though the adrenals of the MAD-treated-intact animals were normal in size, they nevertheless must have been responsible for the grave (in some

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EXPLANATION OF PLATE 1

A.—Intensely nephrosclerotic kidney of MAD-treated-intact rat. Large hyalinized glomerulus and darkly staining hyalin cast in a tubule near upper margin of picture (PAS stain).

B.—Absence of nephrosclerotic changes in MAD-treated-adrenalectomized rat. Glomeruli are normal and tubules show only renotrophic effect, characteristic of testoids (PAS stain).

C.—Intense endothelial proliferation (which forms an endocardial "vegetation") above a hyalinized homogeneous area, in the heart of an MAD-treated-intact rat (H+E stain).

D.—Another region from the myocardium of the animal shown in "C." Note hyalinization underneath the endothelium of the small vessel in the center of the field and widespread myocardial damage around it (H+E stain).

E.—Region comparable to that shown in "D", but without pathologic changes, in an adrenalectomized MAD-treated rat. (H+E stain).

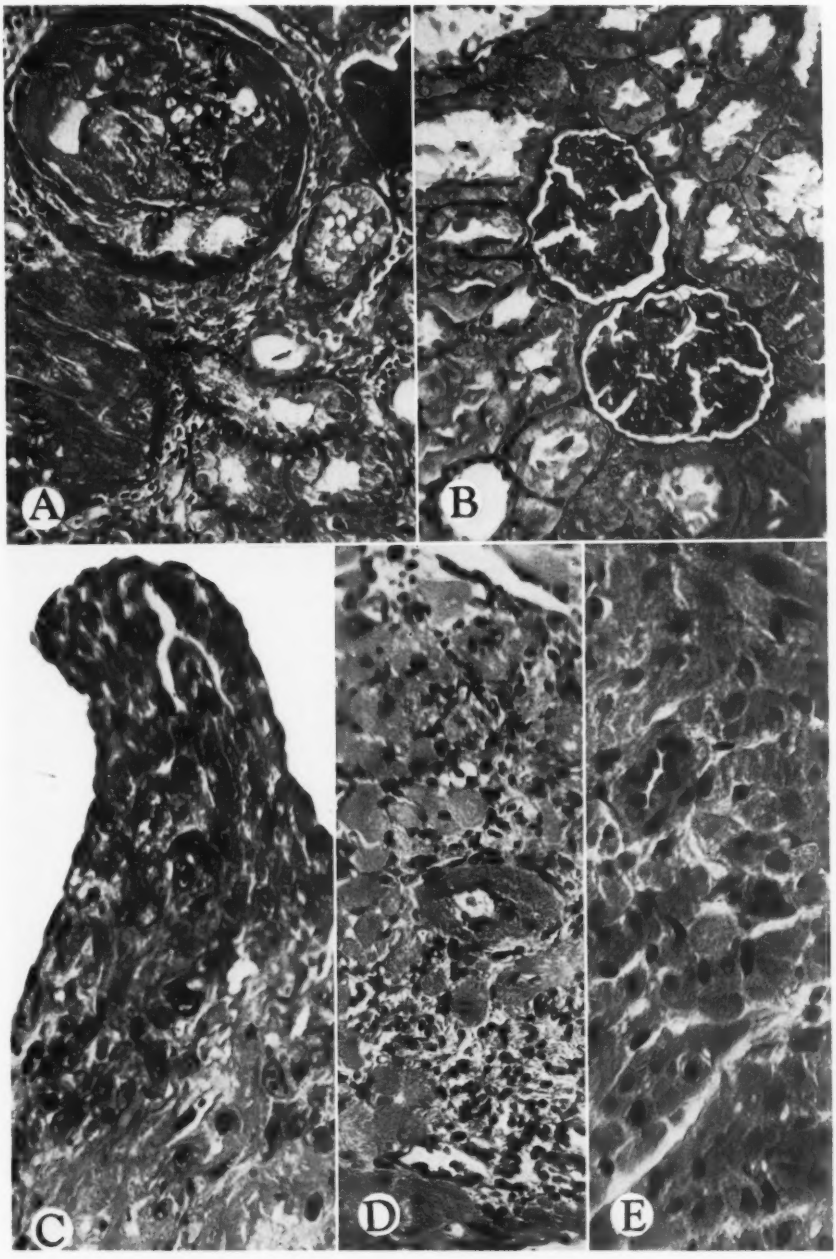


PLATE 1

instances fatal) renal and cardiovascular changes observed in this Group.

The weight of the *spleen* was not markedly altered in any of the groups, while the *thymus* underwent complete atrophy (and hence, could not be weighed) in both the intact and in the adrenalectomized-MAD-treated animals. This shows the possibility of inducing a dissociation among the various effects of this steroid by adrenalectomy. While its nephrosclerotic and cardiovascular actions are dependent upon the integrity of the adrenals, its thymolytic effect is not.

Similarly, the *preputial gands*, which are fairly accurate indicators of testoid hormone actions, showed pronounced stimulation by MAD, both in the presence and in the absence of the adrenals.

Finally, MAD caused a slight but significant involution of the *hypophysis* in the intact, as well as in the adrenalectomized animals, when compared with the corresponding not hormone-treated controls.

*Histologic studies* confirmed the macroscopic observations, as regards the presence of the hyalinosis in the MAD-treated-intact animals, and their absence in all other groups. They also demonstrated the constancy and intensity of the colloid granule formation in the adrenals of the MAD-treated animals. Curiously, granules with similar tinctorial properties have also been noted in the convoluted tubules of the kidney and, occasionally, in some cells of cardiac granuloma tissue. As an incidental and hitherto unexplained finding, we observed (both in intact and in adrenalectomized) MAD-treated rats, patches of pancreatic acinar tissue, which underwent a peculiar transformation into "signet-ring" cells (cf. Plates 1 and 2).

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EXPLANATION OF PLATE 2

A.—Periarteritis nodosa of pancreatic artery in an MAD-treated-intact rat (H+E stain).

B.—Peculiar signet-ring-like degeneration of the pancreatic acinar-cells (H+E stain). This was present in many MAD-treated rats, irrespective of whether they had been adrenalectomized or not.

C.—Hemorrhage and liquification necrosis in the brain of an MAD-treated-intact rat. Note also intense staining of vascular walls, due to beginning hyalin deposition (H+E stain).

D.—Colloid granules (similar to those seen in the adrenal) in a granulomatous nodule, within the heart-muscle of an MAD-treated-intact rat (PAS stain).

E.—Numerous fine colloid granules within the renal tubules of an MAD-treated-intact rat (PAS stain).

F.—Typical colloid-storing region from the inner fasciculata of an MAD-treated rat. Of all organs, the formation of hyalin granules is most evident here. Upper arrow points to cortical cell, with clearly visible cell-membrane and nucleus, in whose cytoplasm small PAS-positive granules begin to appear (just above the nucleus). The lower arrow indicates a cortical cell which is almost completely filled by an enormous dark-staining colloid granule (PAS stain).

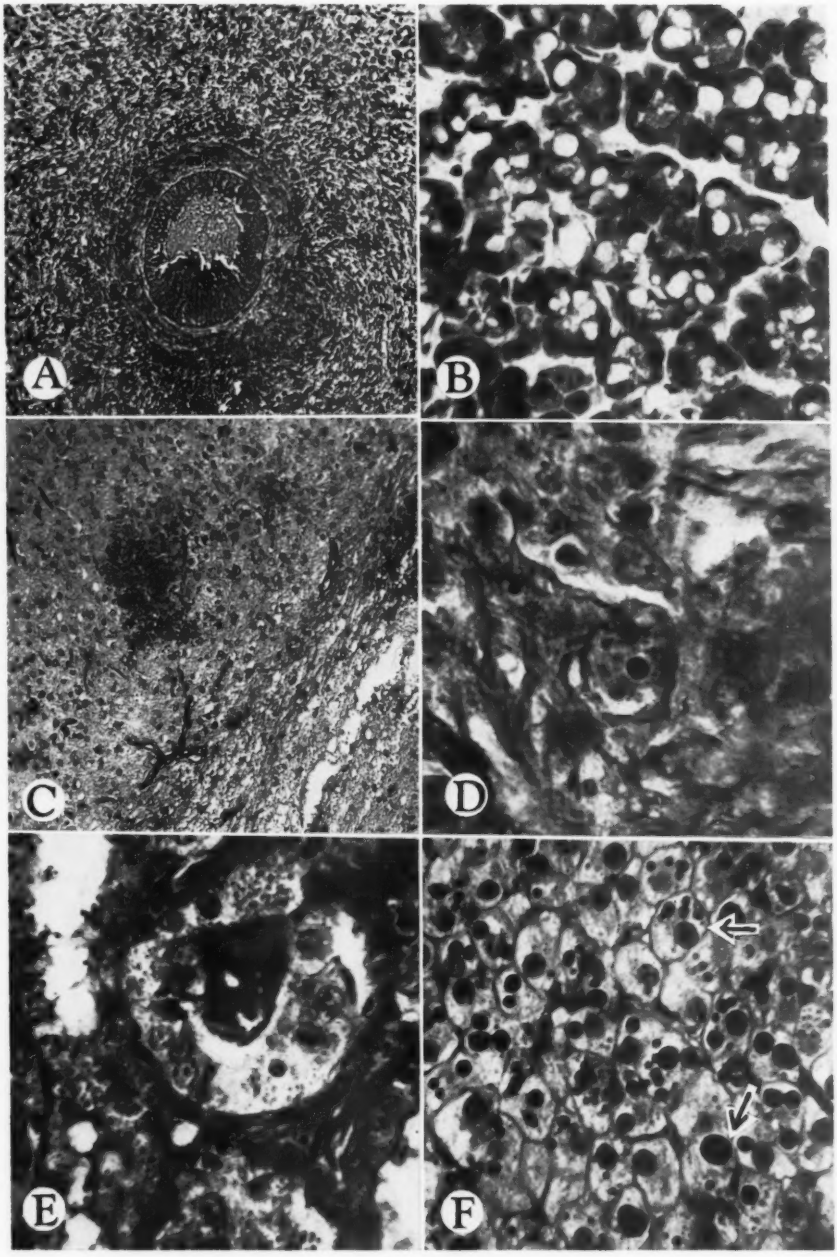


PLATE 2

## DISCUSSION

These observations fully confirm the finding of Skelton (1953), that MAD can produce a syndrome of hyalinosis, with hypertension and polyuria, that is, changes essentially similar to those caused by DCA overdosage. The present investigations give additonal significance to the original observations, in that they clarify the mechanism of this action. It was confusing, at first, to note that a synthetic androgen, such as MAD, could produce pathologic changes, hitherto considered to be characteristic of mineralo-corticoids. By demonstrating that these unexpected actions of MAD are wholly dependent upon the presence of the adrenal glands, the concept of a close relationship between the hyalinosis syndrome and the suprarenals actually gains further support.

It had been noted by others that MAD can stimulate adrenal growth, at least in the sense that it prevents the adreno-cortical atrophy, otherwise produced by cortisone or hypophysectomy in the rat (Winters *et al.*, 1953; Gaunt *et al.*, 1953). It is particularly noteworthy, however, that in our experiments, a syndrome resembling mineralo-corticoid overdosage was produced by MAD, without any increase in adrenal size. Yet, this syndrome was manifestly dependent upon these glands, since it could be prevented by adrenalectomy. In view of these findings we cannot subscribe to the commonly voiced assumption that in man, the absence of a demonstrable adrenal enlargement at autopsy suggests that there was no hypercorticoidism during life. Systematic studies concerning the experimental morphology of the adrenals had suggested, a few years ago, that these glands can respond to diverse stimuli with qualitatively different morphologic responses, such as colloid-granule formation, myeloid or fatty metaplasia, tubule formation, etc., and "that the use of specific stimulators enable us to arrive at what might be called the pharmacology of the adrenal cortex" (Selye and Stone, 1950). The abundant formation of PAS-positive "colloid" granules, under the influence of MAD and related steroids, may well be the morphologic expression of excessive mineralo-corticoid production, although our observations do not prove that these histologic changes are actually the cause of such functional changes.

It also remains to be shown whether the adrenals of MAD-treated rats actually produce an absolute excess of mineralo-corticoids, or whether their hormones merely "condition" the tissues to the hyalinosis-producing effect of MAD. Conditioning influences are undoubtedly important in the pathogenesis of this syndrome. It will be recalled that even a typical mineralo-corticoid, such as DCA, fails to cause hylinosis (nephrosclerosis, myocarditis, periarteritis) in the rat after hypophysectomy (Salgado and Selye, 1952), but it is even more than normally effective in this respect after adrenalectomy (Selye *et al.*, 1944). Thus, there exists a clear-cut dif-

ference between MAD and DCA when these compounds are tested on adrenalectomized rats. Similar comparative studies on hypophysectomized animals are now under way and will be published elsewhere.

The striking difference between the findings reported here and those of earlier investigators (Guillemin and Conti, 1953; Skelton, 1953) is that, in our MAD-treated animals, there was no indication of myeloid metaplasia in the adrenals, MAD caused no excessive local irritation and inflammation at the site of injection, nor did it prove toxic to adrenalectomized animals. Myeloid metaplasia in the adrenals tends to occur especially in the presence of multiple abscesses or extensive tissue-necroses, which are also highly toxic to adrenalectomized rats. It is probable that all these effects, which we did not observe, are interrelated and depend upon the injection technique, rather than upon specific pharmacologic properties of MAD.

## SUMMARY

Experiments on intact rats, sensitized by unilateral nephrectomy and a high sodium intake, confirm earlier observations that methylandrostenediol (MAD) can produce a syndrome of hyalinosis (characterized by nephrosclerosis, periarteritis nodosa and myocarditis, with hyalinized nodules in the heart) accompanied by hypertension and polyuria. All these changes are quite comparable to those which had previously been obtained with mineralo-corticoids, such as DCA.

None of the above mentioned pathologic changes are produced by MAD after bilateral adrenalectomy, in similarly sensitized rats.

The production of hyalinosis by MAD is not associated with any increase in adrenal weight, but is accompanied by a characteristic histologic change, the accumulation of numerous hyalin granules in the adrenocortical cells.

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# ADRENOCORTICOTROPIC SUBSTANCES FROM HUMAN PLACENTA[1]

N. S. ASSALI AND J. HAMERMESZ[2]
With the technical assistance of M. ROSS
*Department of Obstetrics and Gynecology, University of California at Los Angeles, Los Angeles, California*

IT HAS been suggested repeatedly that during pregnancy, there is an increased adrenocortical activity. Venning (1946) found a progressive rise in the excretion of corticoids beginning early in pregnancy and reaching its maximum in the last trimester of gestation. Subsequently, the same author (1954) observed still further increase in the rate of excretion of the salt retaining fraction in association with toxemia of pregnancy. Similar changes were reported by Tobian (1949) and by Lloyd and his coworkers (1952). Gemzell (1953) showed that the blood levels of 17-hydroxicorticosteroids began to rise early in pregnancy and remained high until shortly after delivery. He attributed this steady rise in part to a stimulation of the maternal adrenals by excessive amounts of estrogen present during pregnancy, and to hyperactivity of the fetal adrenals. The latter have also been held responsible for the spontaneous improvement in patients with Addison's disease usually seen during pregnancy (Samuels *et al.*, 1943). However, Day (1948) and Jailer (1950) found low rates of 17-ketosteroids and corticoid-like substances in fetal urine and Klein (1953) observed very low levels of 17-hydroxycorticosteroids in the blood of newborn infants. Jailer further noted that a crude acetone dried placental extract exhibited a certain adrenocorticotropic (ACTH) activity when assayed in newborn intact rats. Tarrantino (1951), Opsahl and Long (1951), Cohen and Kleinberg (1952) and Sulman and Bergman (1953) confirmed Jailer's finding on placental extracts, although the methods used by some of these authors were not specific for assays of ACTH activity.

In previous studies (Assali *et al.*, 1953 and 1954) it was shown that the pregnant woman had a certain tolerance to the action of relatively large doses of either desoxycorticosterone acetate (DCA) or ACTH. It was suggested that this spontaneous tolerance might have been built up by the overproduction of these hormones by the placenta.

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[2] Present address—Hospital das Clinicas, University of Sao Paulo, Sao Paulo, Brazil.

The present study was designed to test this hypothesis by assaying several preparations obtained from placental tissues and from placental blood for ACTH activities. The study also includes comparison of the activity of some of these fractions to that of the standard U. S. P. corticotropin.

## EXPERIMENTAL METHODS

Fresh placentas were collected immediately after delivery; the membranes and the umbilical cords were discarded and the remainder was frozen in dry ice and shipped by air to Armour Laboratories in Chicago.[3] These laboratories processed the following four fractions, which were returned to us in lyophilized form: Fraction A was prepared by the hydrochloric acid-acetone technique from blood contained in the intervillous space; fraction B was prepared from the same material by the oxycellulose technique of Astwood (1951); fraction C was prepared from the dried chorionic villous tissue by the acetic acid-acetone technique, and fraction D was prepared from the same material by the technique used in the preparation of fraction B. All of the four fractions were assayed for ascorbic acid depletion in a similar manner in Sprague-Dawley male hypophysectomized rats (120–160 gm.) delivered by air from Hormone Assay Laboratories, Chicago. The assays were carried out according to Sayer's (1948) technique 24 to 30 hours after hypophysectomy. After the rat had been anesthetized with *Surital* (4 mg./100 gm. body weight), the left adrenal was removed, weighed to the nearest 0.1 mg. and prepared for analysis. Thereafter, one half ml. of saline solution containing the calculated dose of the fraction to be tested was injected in the tail vein. One hour later the right adrenal was dissected and prepared for analysis. The completeness of the hypophysectomy was checked at autopsy and when remnants of pituitary tissue were found, the data on that animal were deleted. The concentration of ascorbic acid in the adrenal tissues was determined by the method of Roe and Kuether (1943).

The potency of fraction D was compared to that of the standard U. S. P. corticotropin using the 2×2 method. Two proportional doses of fraction D and two of the standard were assayed in paired animals on the same day and following the same technique.

## RESULTS

In Figure 1 is compared the percentage of ascorbic acid depletion induced by the injection of a test dose (5 mg./100 gm. body weight) of the 4 different fractions. It can be seen that fractions B and D, which were prepared by the oxycellulose technique, produced an average of 38 and 55% of ascorbic acid depletion respectively, whereas the 2 other fractions were practically inactive.

Table 1 shows detailed data on the assay of fraction D obtained from the study of 79 animals. In it are noted the number of animals employed in testing each dose, the mean adrenal weight and the ascorbic acid concentrations and differences on both sides. Figure 2 shows the arithmetic dose-response relationship of fraction D with the standard deviation of the re-

[3] The authors are indebted to Joseph Fisher and other members of the Armour Research Laboratories for the preparation of the various placental fractions and for the supply of the standard USP corticotropin.

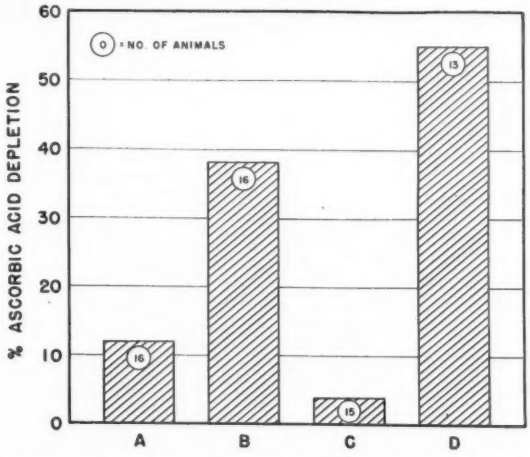


FIG. 1. The response obtained following the injection of 5 mg./100 gm. of body weight of fractions A, B, C, and D. The numbers in the circle contained in each block represent the number of animals employed. It is evident that significant ascorbic acid depletion was induced only by fractions B and D.

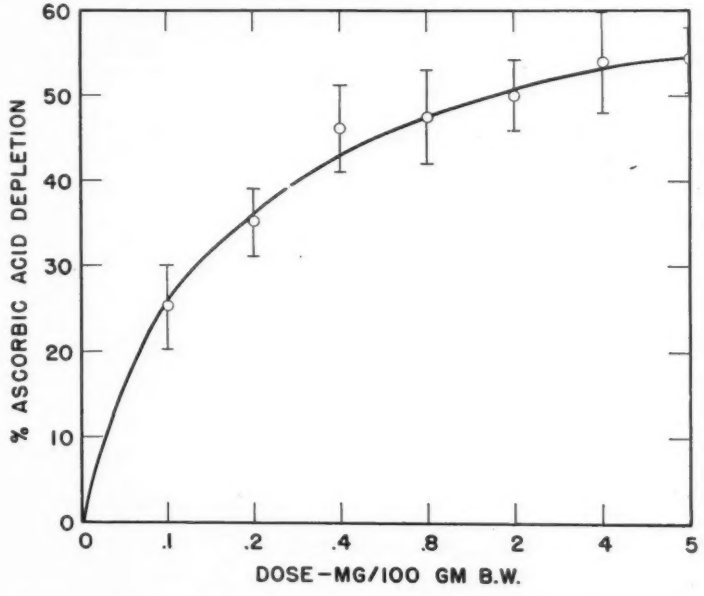


FIG. 2. Dose-response relationship of fraction D obtained from the study of 79 animals. The T connected to each datum represents one standard deviation of the response to each dose. The curve tends to level off after 1 mg. dose and takes the form of an hyperbola.

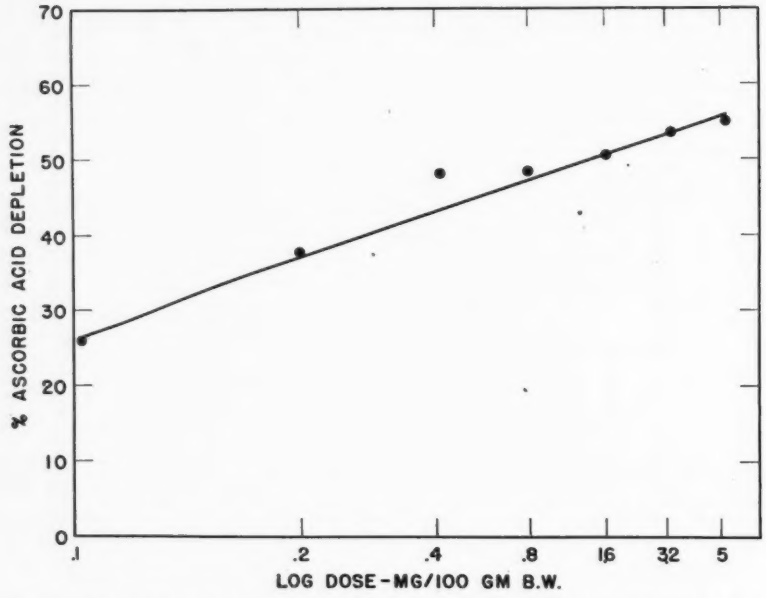


FIG. 3. The arithmetic response is plotted against the log dose of fraction B. A straight line is obtained which connects the majority of points.

sponse to each dose. It is evident from the figures that a progressive ascorbic acid depletion is observed with increasing doses of fraction D. However, the responses to doses above 1 mg. tend to level off, the curve being that of an hyperbola. This curve is almost similar to that obtained by Sayer (1948) in the assay of pure ACTH hormone. When the arithmetic response is plotted against the log dose of fraction D, a straight line is obtained (Fig. 3).

In Figure 4 are plotted the arithmetic responses against the log of 2 proportional doses of fraction D and 2 doses of standard U. S. P. corticotropin.

TABLE 1. DATA ON ASSAY OF FRACTION D

| Dose, mg./100 gm. B.W. | No. rats | Mean adrenal w., mg. | | Ascorbic acid, mg./100 gm. adr. | | Difference, mg. | % |
|---|---|---|---|---|---|---|---|
| | | Left | Right | Left | Right | | |
| 0.1 | 13 | 11.5 | 9.8 | 378 | 284 | 94 | 24.8 ± 6 |
| 0.2 | 14 | 13.8 | 12.5 | 436 | 280 | 156 | 35.7 ± 4 |
| 0.4 | 13 | 10.8 | 11.3 | 403 | 216 | 187 | 46.5 ± 5 |
| 0.8 | 10 | 12.1 | 12 | 438 | 233 | 205 | 47 ± 6 |
| 2 | 6 | 13 | 12.3 | 483 | 240 | 243 | 50 ± 4 |
| 4 | 10 | 12.5 | 12.3 | 460 | 216 | 244 | 53 ± 6 |
| 5 | 13 | 11.4 | 10.6 | 500 | 227 | 273 | 55 ± 4 |

TABLE 2. DATA ON THE ASSAYS OF FRACTION D AND THE STANDARD USP CORTICOTROPIN

| Preparation | Dose, 100 gm. b.w. | No. rats | Ascorbic acid, mg./100 gm. adrenal | | % depletion |
|---|---|---|---|---|---|
| | | | Left | Right | |
| Fraction D | 0.1 mg. | 8 | 402 | 300 | 25.4 |
| | 0.2 mg. | 8 | 509 | 318 | 37.5 |
| Standard | 0.5 mu. | 8 | 433 | 403 | 7.0 |
| | 1.0 mu. | 8 | 455 | 316 | 30.5 |

The lines are not exactly parallel which makes the calculation of the potency ratio somewhat difficult. Nevertheless, it can be seen by simple inspection that the activity of 0.15 mg. of fraction D is approximately equivalent to that of 1 milliunit of the standard. Table 2 shows the data on the com-

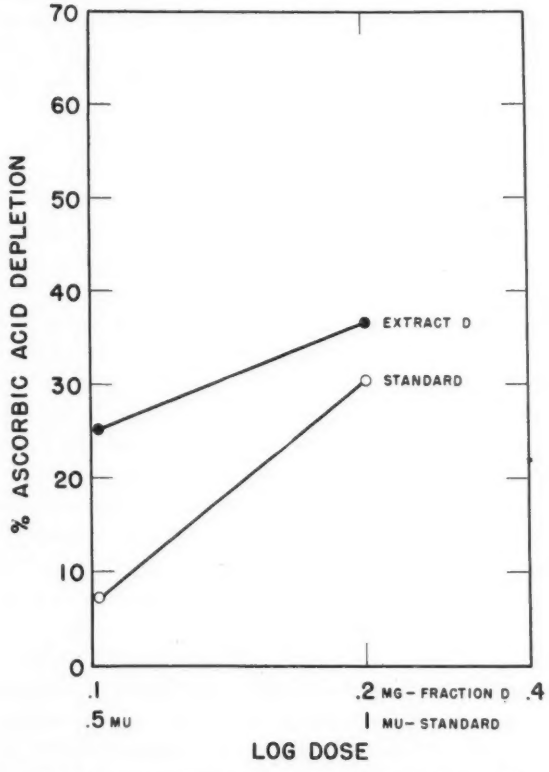


FIG. 4. The arithmetic responses are plotted against the log of 2 proportional doses of fraction D and 2 doses of the standard U. S. P. corticotropin (0.1 and 0.2 mg. of fraction D and 0.5 and 1 mu. of the standard). The lines are not parallel.

parative assays of the standard U. S. P. corticotropin and fraction D. Calculation of the yield of the placental tissue revealed that 1 mg. of fraction D may be obtained from 220 mg. of dried chorionic villi, which yields approximately 6.6 milliunits of ACTH-like substances.

## DISCUSSION

It has been well known that during pregnancy, besides an increased tendency for water and electrolyte retention, there occur frequently certain metabolic and physiologic changes which simulate overactivity of the pituitary-adrenocortical axis. The increased excretion of adrenal metabolites, as well as the elevated blood levels of corticosteroid hormones and the improvement in certain collagen diseases, attest to an increased production of adrenal hormones during normal pregnancy. Some authors have attributed this increase to a stimulation of the maternal adrenals by the excessive amount of estrogens present in the maternal blood. The present data indicate the likelihood that the ACTH-like substances produced in large quantity by the placenta are the main factor responsible for the adrenal stimulation.

It appears evident from these studies and those made by others (Johnson, 1952) on the production of corticosteroid substances and other hormones by the chorionic villi that the placenta has multiple functions. Besides its role as a vital link between mother and fetus, it serves to provide a certain degree of biological autonomy to the reproductive process through its continuous production of most of the hormones which are essential for the protection of the growth phenomena. Under these circumstances, it is reasonable to assume that certain hormonal activities of the adenohypophysis may be markedly depressed during the pregnant state, and that the placenta may take over a major part of the pituitary functions.

As to the natural tolerance exhibited by the pregnant woman to the action of exogenous DCA and ACTH, it seems likely in the light of the present data that the doses administered were insufficient to raise the blood level of these hormones and to cause significant physiologic changes since these patients already had an elevated blood level. It is not possible to state at the present time whether larger doses might have induced some of the expected metabolic changes.

Of interest is the marked difference in the activities of the fractions prepared by the usual techniques of acid-acetone and those prepared by the oxycellulose technique. The results of this study support those of Astwood (1951) and indicate that the use of the oxycellulose procedure presents an efficient method for purification of crude extracts containing adrenocorticotropic substances.

In regard to the site of production of the ACTH-like substances in the

placenta, it seems probable that the chorionic villi are the main source, since the fraction obtained from these tissues showed the greatest activity. However, since the blood contained in the intervillous space also induced a significant ascorbic acid depletion, the possibility still remains that the chorionic villi are merely storing rather than actively producing these hormones. Plans are being made to study this possibility.

SUMMARY

Four fractions obtained from human placental tissue and from blood contained in the intervillous space were assayed for adrenocorticotropic activity by the ascrobic acid depletion method.

Fractions D and B, which were prepared by the oxycellulose technique from dried chorionic villi and from intervillous blood respectively, showed a striking corticotropic activity. The activity of 0.15 mg. of fraction D was found to be comparable to that of 1 milliunit of the standard USP corticotropin. The significance of these findings with regard to the physiologic changes of pregnancy has been discussed.

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# BIOASSAY OF MINERALOCORTICOIDS: RELATIONSHIP OF STRUCTURE TO PHYSIOLOGICAL ACTIVITY

B. J. AXELRAD, J. E. CATES, B. B. JOHNSON, AND J. A. LUETSCHER, JR.

*Department of Medicine, Stanford University School of Medicine, San Francisco, California*

ADRENAL cortical extracts and human urine contain a corticosteroid of great potency measured by its effect on sodium and potassium excretion and on the survival of adrenalectomized animals.

Wintersteiner and Pfiffner (1936) found that very small doses of the "amorphous fraction" of adrenal cortical estract maintained life after adrenalectomy. Simpson and Tait (1953), Mattox, Mason, and Albert (1953), and Knauff, Nielson and Haines (1953) have prepared crystalline materials of very similar properties and of high activity on sodium and potassium excretion. The chemical structure of this mineralocorticoid has recently been described as the 11–18 hemiacetal of 18-aldehydo-corticosterone by Simpson, Tait, Wettstein, Neher, v. Euw, Schindler, and Reichstein (1954), who have suggested the definitive term, aldosterone.

Grollman and Firor (1932) demonstrated that extracts of human urine prolonged the survival of adrenalectomized animals. The urine of edematous patients with lipemic nephrosis, heart failure, and hepatic cirrhosis contains increased quantities of a sodium-retaining corticoid, which is presumed to be aldosterone on the basis of a number of physical, chemical, and pharmacological properties (Luetscher and Johnson, 1953, 1954a, b; Sala and Luestcher, 1954).

In the search for corticosteroids with sodium-retaining activity, a number of new compounds have been generously supplied by the chemists responsible for their synthesis. The bioassays furnish some new information on the relationship between chemical structure and a specific type of physiological activity.

## MATERIALS AND METHODS

Each crystalline steroid was furnished through the generosity of the chemist or pharmaceutical firm responsible for its synthesis. The source of each compound is identified in the tables. Samples were weighed and dissolved in redistilled ethanol 95%. The concentration was checked by measurement of ultraviolet light absorption near 240 mμ. characteristic of compounds with the $\Delta^4$, 3-keto grouping, or by the reduction of neotetrazolium by the α-ketol side chain.

*Bioassay:* The effect on sodium and potassium excretion was measured in adrenalectomized rats by the technique of Luetscher and Deming (1951) as modified by Johnson

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(1954). The results are expressed as the number of micrograms of desoxycorticosterone acetate (DCA) which would produce the change observed after administration of the test material. Increased sodium excretion, which is observed after the administration of large doses of cortisone, hydrocortisone, corticosterone, or 11-dehydrocorticosterone, is reflected by an estimate of activity less than zero when the result is expressed as DCA equivalent.

Only minor differences were found between estimates of dosage based on sodium output and those based on potassium to sodium ratio. The latter estimate has been used for further calculation because of its greater reliability. The potency of each compound relative to DCA has been calculated by dividing the estimate of effect expressed as milligrams of DCA by the dosage of the compound in milligrams administered. A compound equal to DCA in activity would have a potency of 1.0, while an inert compound's potency would be zero. When significant activity was demonstrated, the 95% confidence limits (Johnson, 1954) are indicated in the calculation of potency. When the 95% confidence limits of an assay included zero dosage, potency is expressed as less than the highest probable value. When a compound significantly increased the excretion of sodium, the potency is indicated as less than zero.

*Screening test:* The effects of large doses of certain compounds were first studied by this less sensitive test. It differed from the bioassay, in the following respects. The screening tests was carried out on one day, comparing 5 test rats with 5 simultaneous controls. Adrenalectomized male rats weighing about 150 gm. were used. The intraperitoneal injection on the test day consisted of 5 ml. of 0.45% sodium chloride solution.

The reliability of the screening test was examined by statistical methods devised by Dr. L. E. Moses. Seven sets of control rats, studied on different test days, showed insignificant differences in sodium output or in urine potassium to sodium ratio. Accordingly, these rats were treated as one group, to which results for the various steroids were compared by the Mann-Whitney test (1947). Significance points were calculated from the table of White (1952). The sensitivity of the test is indicated by the response to several doses of DCA (Table 2).

Interpretation of borderline positive results of the screening test must be made with caution. There is only a very small risk of failing to find activity in a compound as active as DCA, since the doses employed (200 $\mu$g. per rat when solubility permitted and no less than 75 $\mu$g. in any case) were much larger than the dosage of DCA which could be regularly detected.

## RESULTS

Table 1 indicates compounds of insignificant activity in the dosage used in the screening test. The saturated compounds numbered I to IX failed to produce the distinctive effects on electrolyte excretion manifested by their $\Delta^4$, 3-keto analogues. The effect of the $\Delta^1$ unsaturation can not be assessed in substance X because dosage was limited by relative insolubility.

Table 2 summarizes the effects of certain compounds on sodium and potassium excretion in the screening test. Effects on electrolyte excretion, comparable to those produced by 10$\mu$g. or more of DCA, were noted after large doses of 6$\beta$-hydroxy-progesterone (XIV), the $\Delta^1$ analogue of DCA (XVII), and $\Delta^5$-pregnenediolone (XVIII). Changes of doubtful significance were observed after similar dosage of three other substances (XIII, XV,

TABLE 1. COMPOUNDS SHOWING INSIGNIFICANT ACTIVITY IN SCREENING TEST[1]

| Number | Class and name | Dose tested μg. |
|---|---|---|
| | *Saturated, 3-hydroxyl* | |
| I | Pregnane-3β,21-diol-20-one (diacetate) | 200 |
| II | Pregnane-3β,21-diol-11,20-dione (diacetate) | 200 |
| III | Pregnane-3β,17α,21-triol-20-one (diacetate) | 200 |
| IV | Pregnane-3α,12α,21-triol-20-one (triacetate) | 200 |
| V | Allopregnane-3β,21-diol-20-one (diacetate) | 200 |
| | *Saturated, 3-keto* | |
| VI | Pregnan—21-ol-3,20-dione (acetate) | 200 |
| VII | Allopregnan—21-ol-3,20-dione (acetate) | 162 |
| VIII | Allopregnane-17α,21-diol-3,20-dione (acetate) | 76 |
| IX | Allopregnane-17α,21-diol-3,11,20-trione (acetate) | 84 |
| | *$\Delta^1$,3-keto* | |
| X | $\Delta^1$-allopregnene-17α,21-diol-3,20-dione (acetate) | 75 |
| | *Other* | |
| XI | $\Delta^{3,5}$-pregnadiene-3,17α,21-triol-11,20-dione (diacetate) | 200 |

[1] Chemical Specialties, Syntex, furnished these compounds.

and XVI). Five materials were subjected to further testing by quantitative bioassay.

Table 3 gives the results of the quantitative bioassays. The preparations from adrenal cortical extract (aldosterone) and from human urine (sodium-retaining corticoid) showed activity respectively 30 and 20 times that of DCA.

The 9α-chloro derivatives of cortisone and hydrocortisone acetates (XIX, XX) were considerably more active than DCA. 19-nor-desoxycorticosterone (XXI) and $\Delta^{9(11)}$ desoxycorticosterone (XXIII) appeared to be slightly more active than DCA. All other compounds tested showed activity less than one fifth that of DCA. The three derivatives of 11-de-

TABLE 2. COMPOUNDS AFFECTING SODIUM AND POTASSIUM EXCRETION IN SCREENING TEST

| Number | Name | Dose μg. | Fall in sodium output | Fall in Na/K ratio |
|---|---|---|---|---|
| XII | $\Delta^4$-pregnen—21-ol-3,20-dione (acetate)[1] (DCA) | 5 | 0 | 0 |
| | $\Delta^4$-pregnen—21-ol-3,20-dione (acetate)[1] (DCA) | 10 | + | ++ |
| | $\Delta^4$-pregnen—21-ol-3,20-dione (acetate)[1] (DCA) | 25 | + | ++ |
| | $\Delta^4$-pregnen—21-ol-3,20-dione (acetate)[1] (DCA) | 25 | + | ++ |
| | $\Delta^4$-pregnen—21-ol-3,20-dione (acetate)[1] (DCA) | 25 | ++ | ++ |
| | $\Delta^4$-pregnen—21-ol-3,20-dione (acetate)[1] (DCA) | 200 | ++ | ++ |
| XIII | $\Delta^4$-pregnene-3,20-dione[3] | 200 | + | 0 |
| XIV | $\Delta^4$-pregnen—6β-ol-3,20-dione[2] | 200 | ++ | ++ |
| XV | $\Delta^4$-pregnene-2β,21-diol-3,20-dione (diacetate)[2] | 170 | 0 | + |
| XVI | $\Delta^4$-pregnene-3β,6β,21-triol-20-one (triacetate)[2] | 200 | + | 0 |
| XVII | $\Delta^1$-allopregnen—21-ol-3,20-dione (acetate)[2] | 133 | + | ++ |
| XVIII | $\Delta^5$-pregnene-3β,21-diol-20-one[2] | 200 | ++ | ++ |

[1] Ciba Pharmaceutical Products, Dr. C. R. Scholz.
[2] Chemical Specialties, Syntex, Dr. J. R. Mote.
[3] Schering Corporation, Dr. E. Henderson.
Effect: 0 insignificant
+ $P \leqq .05$
++ $P \leqq .01$

TABLE 3. COMPOUNDS STUDIED BY QUANTITATIVE BIOASSAY

| Number | Class and name | Dose μg. | Response | | Relative potency DCA = 1.0 |
|---|---|---|---|---|---|
| | | | Sodium | K/Na | |
| | | | DCA Equiv. μg. | | |
| | *Related to Aldosterone* | | | | |
| | Aldosterone[9] | 0.16 | 7.9 | 5.9 | 37 ± 15 |
| | | 0.36 | 11.8 | 9.7 | 28 ± 6 |
| | Sodium-Retaining Corticoid[10] | 0.21 | — | 4.2 | 20 ± 11 |
| | | 0.52 | 7.7 | 8.8 | 17 ± 4 |
| | *9α-Chloro-Substituted* | | | | |
| XIX | 9α-chloro-cortisone (ac)[2] | 1.0 | 7.5 | 6.4 | 6.4 ± 2.3 |
| XX | 9α-chloro-hydrocortisone (ac)[2] | 1.0 | — | 5.2 | 5.2 ± 2.5 |
| | | 1.5 | 9.1 | 8.1 | 5.4 ± 1.6 |
| | *Related to 11-desoxycorticosterone* | | | | |
| XXI | 19-nor-desoxycorticosterone[8] | 3.8 | 6.7 | 4.8 | 1.3 ± 0.6 |
| | | 4.2 | 8.3 | 7.2 | 1.7 ± 0.6 |
| XXII | $\Delta^4$-pregnene-19,21-diol-3,20-dione[1] | 25 | 1.0 | 1.1 | ≦0.13 |
| XXIII | $\Delta^{4,9(11)}$-pregnadien-21-ol-3,20-dione (ac)[2] | 4.5 | 6.7 | 5.2 | 1.2 ± 0.6 |
| XV | $\Delta^4$-pregnene-2β,21-diol-3,20-dione (diac)[3] | 100 | 2.5 | 2.1 | ≦0.04 |
| XXIV | $\Delta^4$-pregnene-6α,21-diol-3,20-dione[1] | 25 | 3.3 | 2.1 | ≦0.17 |
| XXV | $\Delta^4$-pregnene-6β,21-diol-3,20-dione[1] | 25 | −2.6 | −2.6 | <0* |
| XXVI | $\Delta^4$-pregnene-16α,21-diol-3,20-dione[4,2] | 100 | −0.8 | −2.6 | <0* |
| XVII | $\Delta^5$-allopregnen-21-ol-3,20-dione (ac)[7] | 25 | −0.2 | 1.3 | ≦0.15 |
| | *Related to Reichstein's Substance S* | | | | |
| XXVII | $\Delta^4$-pregnene-17α,21-diol-3,30-dione[4] | 200 | 8.1 | 6.2 | 0.03 ± .01 |
| XXVIII | $\Delta^{4,9(11)}$-pregnadiene-17α,21-diol-3,20-dione (ac)[2] | 35 | 1.2 | 1.0 | ≦0.09 |
| XXIX | $\Delta^4$-pregnene-6α,17α,21-triol-3,20-dione[1] | 50 | 2.0 | 1.8 | ≦0.08 |
| XXX | $\Delta^4$-pregnene-6β,17α,21-triol-3,20-dione[1] | 50 | −3.4 | −2.5 | <0* |
| XXXI | $\Delta^4$-pregnene-17α,20β,21-triol-3-one[3] | 50 | −1.7 | −0.9 | ≦0.03 |
| XXXII | Allopregnane-3β,17α,21-triol-20-one[3] | 10 | −1.3 | −0.6 | ≦0.16 |
| | *Related to Progesterone* | | | | |
| XIII | $\Delta^4$-pregnene-3,20-dione[5] | 200 | 0.3 | 0.8 | ≦0.01 |
| XIV | $\Delta^4$-pregnen-6β-ol-3,20-dione[7] | 100 | 1.5 | −0.2 | ≦0.02 |
| XVIII | $\Delta^5$-pregnene-3β,21-diol-20-one[7] | 50 | 1.2 | 3.0 | 0.06 ± .05 |
| | | 200 | 5.9 | 5.0 | 0.03 ± .02 |

Compounds were furnished through the kindness of:
[1] Dr. M. Ehrenstein, University of Pennsylvania (Herzig and Ehrenstein, 1951; Florey and Ehrenstein, 1954; Barber and Ehrenstein, 1954).
[2] Dr. J. Fried, Squibb Institute for Medical Research (Fried and Sabo, 1953).
[3] Dr. T. F. Gallagher, Sloan-Kettering Institute (Kritchevsky and Gallagher, 1951).
[4] Dr. W. J. Haines, Upjohn Company.
[5] Dr. E. Henderson, Schering Corporation.
[6] Dr. H. Hirschmann, Western Reserve University (Hirschmann, Hirschmann, and Farrell, 1953).
[7] Dr. J. Mote, Syntex-Foundation Laboratories.
[8] Dr. G. Rosenkranz, Chemical Specialties—Syntex S. A. (Sandoval and co-workers, 1953; Sondheimer and co-workers, 1953).
[9] Mrs. S. A. Simpson and Dr. J. F. Tait, Middlesex Hospital (Simpson and co-workers, 1953).
[10] Prepared in this laboratory (Luetscher and Johnson, 1954a) (ac or diac) as mono- or di-acetate.
* Significantly decreased K/Na ratio.

soxycorticosterone with hydroxyl groups at 6β (XXV), 16α (XXVI), or 6β and 17α positions (XXX) increased the excretion of sodium more than that of potassium. This effect is similar to that observed with hydrocortisone, corticosterone, and their 11-dehydro derivatives under the conditions of this bioassay (Johnson, 1954).

## DISCUSSION

The relationship between chemical structure and physiological action of adrenal cortical hormones has been reviewed by Heard (1948). The observed effects on electrolyte excretion generally support Heard's conclusions and add new information.

Saturation of the 4,5-ethylenic bond and of the 3-ketone group results in a gross reduction of physiologic activity (Compounds I–V), both in the

allo—and normal series, with the 3-hydroxyl group in the $\beta$-position. Reichstein (1936) found two related compounds inert in the Everse-de Fremery test, although some life maintaining action has been observed by Kuizenga and Cartland (1939).

Saturation of the 4,5-bond without reduction of the 3-ketone group results in a similar loss of activity (Compounds VI–IX), as noted by Mason, Hoehn, McKenzie, and Kendall (1937) and by Wettstein and Hunziker (1940).

Unsaturation at the 1,2-position (with the 3,4-bond saturated) results in a considerable loss of activity (Compound XVII). $\Delta^5$-pregnenediolone has appreciable activity only in relatively large dosage, as previously reported (Selye, 1941).

Unsaturation at 9, 11 may enhance activity, since Compound XXIII appears to be slightly more active than DCA, as noted by Shoppee and Reichstein (1943).

19-nor-desoxycorticosterone (XXI), prepared by Sandoval and others (1953), is more active than DCA. Ehrenstein (1944) reported less activity in a compound differing only in stereochemical configuration.

The introduction of oxygen in various positions uniformly reduced the activity of desoxycorticosterone. 6$\alpha$-Hydroxy-desoxycorticosterone (XXIV) had an activity about one-tenth that of DCA. Still greater reduction of activity was noted with the 2$\beta$, 17$\alpha$, and 19-hydroxy derivatives of desoxycorticosterone (XV, XXVII, XXII). The 11$\alpha$- and 12$\alpha$-hydroxy derivatives have been reported to have a low order of activity (Gallagher, 1946; Fuchs and Reichstein, 1943), like the 12-keto-compound. The 11$\beta$-hydroxy and 11-keto compounds, corticosterone and 11-dehydro-corticosterone, are inactive at low doses and cause increased sodium excretion at higher doses in the test used (Johnson, 1954). Increased sodium excretion also followed the administration of 6$\beta$- or 16$\alpha$-hydroxy-desoxycorticosterone (XXV, XXVI). If an additional hydroxy substitutent is added at C-17 the sodium-retaining action is further weakened (Compounds XXVIII, XXIX, XXX and other materials studied by Thorn, Engel, and Lewis, 1941; Johnson, 1954).

The 9$\alpha$-chloro derivatives of cortisone and hydrocortisone (XIX, XX: Fried and Sabo, 1953) had the highest sodium-retaining activity of all the artificial compounds tested. Their activity is almost certainly related to the 9$\alpha$-chloro-substituent (Borman and Singer, 1954).

Modifications of the $\alpha$-ketol side chain resulted in loss of activity. Progesterone (XIII) induced trivial effects at relatively high dosage, although its ability to prolong life is recognized (Gaunt and Hays, 1938). 6$\beta$-hydroxy-progesterone (XIV) in a dose of 200 $\mu$g. showed surprising activity in male rats (Table 2), but the 100 $\mu$g. dosage had little effect on the fe-

male rats used in the bioassay. The electrolyte activity of Reichstein's substance S (XXVII) is lost when the 20-carbonyl group is reduced (XXXI). Desoxycorticosterone shows a similar loss of activity when the 20-keto group is reduced (Steiger and Reichstein, 1938).

These observations allow some tentative generalizations concerning the effect of chemical structure on electrolyte activity of adrenal cortical hormones and related compounds. The potency of 11-desoxycorticosterone is diminished by reduction of the $\Delta^4$, 3-keto-structure, by changes in the $\alpha$-ketol side chain, and by the introduction of oxygen in most of the positions studied. Unsaturation at the 9,11-position, absence of the angular methyl group from $C_{10}$, or the introduction of chlorine on $C_9$ tends to increase sodium-retaining activity.

The high potency of aldosterone appears to be related to the formation of a $\gamma$-hemiacetal between the 11$\beta$-hydroxy and 18-aldehyde functions. The structure responsible for the sodium-retaining activity is unusually sensitive to acetylation. Grundy, Simpson, Tait and Woodford (1952) noted that the diacetate of aldosterone has a low activity on bioassay; but the activity may reappear when the acetate is hydrolyzed. We have observed a similar reduction of activity when preparations of sodium-retaining corticoid have been acetylated. These results may be compared with the bioassays of the monoacetates of desoxycorticosterone, cortisone and hydrocortisone, which give results indistinguishable from the corresponding free compound.

SUMMARY

A series of 32 compounds, related in structure to the adrenocortical hormones, have been tested for effect on excretion of sodium and potassium in adrenalectomized rats.

Most modifications of the structure of 11-desoxycorticosterone result in reduced sodium-retaining activity.

Four synthetic compounds of high activity have in common the basic structure of 11-desoxycorticosterone with modifications about carbons 9 or 10. The most potent artificial compounds studied are the 9$\alpha$-chloro derivatives of cortisone and hydrocortisone.

Aldosterone, isolated from adrenal cortical extract, and a corticosteroid presumed to be aldosterone, extracted from human urine, are considerably more potent than any of the other compounds studied.

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# A CLINICALLY SIGNIFICANT STEROIDOGENIC ASSAY OF CORTICOTROPIN (ACTH) ADMINISTERED EXTRAVASCULARLY TO HUMAN SUBJECTS AND TO GUINEA PIGS[1]

GRANT W. LIDDLE,[2] DONALD ISLAND, JEROME CORNFIELD, AND PETER H. FORSHAM

*Metabolic Unit for Research in Arthritis and Allied Diseases, University of California School of Medicine, San Francisco, California*

*and*

*The National Institutes of Health, Bethesda, Maryland*

## INTRODUCTION

IN DEVELOPING what was to become the official method for assaying corticotropin (ACTH) preparations, Sayers, Sayers, and Woodbury (1948) found that more uniform results were obtained during their assays if the ACTH was administered intravenously rather than by extravascular routes. Thus, when ACTH came into clinical use the potencies of various clinical preparations were assayed in terms of their capacity, when administered intravenously to hypophysectomized rats, to cause depletion of adrenal ascorbic acid content. Most of the ACTH administered clinically, however, has been given by the intramuscular route. Soon after the introduction of ACTH into clinical medicine it was reported by Gordon, Kelsey, and Meyer (1951) that the available ACTH preparations were many times more potent, judged by clinical indices, when administered as prolonged intravenous infusions than when administered intramuscularly. It then became apparent from the studies of Forsham, Renold, and Frawley (1951) that the explanation for this discrepancy was to be found in the fact that ACTH preparations were inactivated locally by tissues when administered extravascularly and, furthermore, that this tissue inactivation varied greatly from one ACTH preparation to another as well as from one human recipient to another. In an effort to reduce extravascular in-

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[2] U. S. Public Health Service, Post-doctorate Fellow in Arthritis and Metabolic Diseases. Present address: The Clinical Center, National Heart Institute, Bethesda 14, Maryland.

activation, many of the manufacturers of ACTH adopted better methods of preparation patterned chiefly after the method first described by Payne, Raben and Astwood (1950). The problem is far from solved from the stand point of clinician, however, since it is still easy to demonstrate eight-fold differences in potency among different ACTH preparations, which are labelled as having identical potency on the basis of current assay procedures. A logical method of assaying ACTH, as far as the clinician is concerned, would be one predicting accurately the steroidogenic potency of a given ACTH preparation following intramuscular administration.

The following studies were carried out to determine the feasibility of assaying ACTH under conditions which simulate the essential circumstances under which ACTH is used clinically. Assays were carried out in human subjects because the problem to be solved in this study was primarily a clinical one. Comparable assays were carried out in guinea pigs to determine whether results having clinical relevance could be obtained in a suitable laboratory animal.

## METHODS

### *ACTH Preparations*

The preparations of ACTH employed in this study were provided by Armour Laboratories and by the Upjohn Company and were comparable in quality to ACTH preparations which have been commercially available in the past three years. Labelled potency, expressed in terms of U.S.P. units, had been determined previously by the method of Sayers, Sayers, and Woodbury (1948). Preparations #198 and #284 were purified by the method of Payne, Raben and Astwood (1950). Preparations #16 and #K53906 were salt fractionation products. All preparations were provided in lyophilized form and were suspended in a standard vehicle of sterile 16% gelatin immediately before injection.

### *Analysis of Urinary* 17*-Hydroxycorticoids*

"17-hydroxycorticoid" as used in this paper refers to those compounds of adrenal-cortical origin extractable from urine with butanol which, when treated with phenylhydrazine-sulfuric acid, give maximum absorption at 410 millimicrons. The method employed was a modification of the method of Reddy, Jenkins and Thorn (1952). Since a butanol extract of urine often contains appreciable quantities of contaminants which are chromogenic when subjected to the "Porter-Silber" reaction, it has been necessary to employ a number of intermediate procedures designed to decrease the contaminants maximally without resulting in serious decreases in recovery of corticoids.

*Reagents:*

1. Butanol, reagent grade.
2. "Dilute sulfuric acid:" Sulfuric acid, C. P., 310 ml. plus water, 190 ml.
3. "Color reagent:" 80 mg. Phenylhydrazine-HCl in 100 ml. "dilute sulfuric acid." (Phenylhydrazine-HCl previously recrystallized four times from ethanol).
4. Absolute methanol, redistilled from 2, 4 dinitrophenylhydrazine.
5. Sodium sulfate, anhydrous, Cenco technical grade.
6. 1 M Potassium carbonate (made from reagent grade $K_2CO_3$, anhydrous).

*Standards:*

Five mg. of hydrocortisone (or 5.57 mg. of hydrocortisone acetate) dissolved in 50 ml. absolute methanol as stock solution (100 μg. per ml.) was diluted with methanol to give standards containing 20 and 10 μg. per ml. respectively.

*Butanol Extraction:*

A 10 ml. aliquot of urine was acidified with 5% sulfuric acid to pH 2.5 (human urine) or 3.5 (guinea pig urine). The latter pH was chosen because lower pH resulted in grossly higher urinary pigment blanks. The acidified urine was extracted three times with 1 volume of butanol. The butanol extracts were combined, and water was removed by the addition of anhydrous sodium sulfate. In order to decrease the "urine blank" and to eliminate interfering acidic and phenolic components, the extract was washed with 1/20 volume of 1 M potassium carbonate for 30 seconds. Immediately (any delay was found to result in loss of corticoids) sufficient anhydrous sodium sulfate was added to remove the aqueous base, and the extract was filtered through glass wool. An aliquot of the filtrate was transferred to an Erlenmeyer flask, warmed to 50° C., and evaporated to dryness under a stream of air.

*Colorimetry:*

The extract residue was dissolved in 3–5 ml. of methanol. A 1 ml. aliquot was added to each of two duplicate pyrex tubes ("e" and "f" in the table below). To one of these tubes was added 5 ml. of "dilute sulfuric acid," and to the other 5 ml. of the "color reagent." In similar manner blanks and standards were prepared as indicated below. The tubes were stoppered with "Parafilm"-protected corks, mixed by inversion and were placed in a water bath at 60° C. ±1° for 20 minutes. They were then cooled for 3 minutes, and the optical densities at 410 millimicrons were determined using a Beckman DU spectrophotometer.

The tubes were set up as follows:

| a. Colorimeter Blank | b. Reagent Blank | c. Standard Blank | d. Standard (10–20 μg.) | e. Urine "apparent corticoid" | f. Urine Blank |
|---|---|---|---|---|---|
| 1 ml. methanol<br>5 ml. $H_2SO_4$ | 1 ml. methanol<br>5 ml. color reagent | 1 ml. standard<br>5 ml. $H_2SO_4$ | 1 ml. standard<br>5 ml. color reagent | 1 ml. extract<br>5 ml. color reagent | 1 ml. extract<br>5 ml. $H_2SO_4$ |

*Sample Calculation:*

(Micrograms of "17-hydroxycorticoids" per urine specimen.)

$$\frac{(e-b)-(f-a)}{(d-b)-(c-a)} \times \frac{\text{urine vol.}}{\text{sample vol.}} \times \text{butanol dilution} \times \text{methanol dilution} \times \mu\text{g. of standard}$$

*Human Assay Procedure:*

Human volunteers, both male and female, between the ages of 20 and 50 were employed as subjects. Single doses of ACTH were administered intramuscularly at least three days apart in order to avoid progressive enhancement of adrenal responsiveness to ACTH. Each subject received three different dose levels of each of the ACTH preparations used in any one comparative study. The dosage range for each preparation was established empirically so that the lowest of the three dose levels gave an appreciable

increase in corticoid excretion while the highest was still within the linear portion of the log dose-response curve. Complete urine collections were made for the 24-hour period over 5 ml. of 3% thymol in glacial acetic acid, and 17-hydroxycorticoids were determined following each injection.

*Guinea Pig Assay Procedure:*

Male guinea pigs weighing between 300 and 500 grams. were used. The guinea pig colony was divided into groups of 8 animals each. All of the individual guinea pigs in a given group received identical doses of ACTH. The individual specimens of urine for any one group of 8 animals were pooled to form a single specimen for steroid analysis. During assays the animals were placed in metabolic cages where they were maintained on an *ad lib.* diet of rabbit pellets and water. A coarse conical screen was used to deflect food and feces away from the collecting area. Urine was collected in a pyrex funnel draining into an Erlenmeyer flask containing 1 ml. of 3% thymol in glacial acetic acid as a preservative. One specimen of urine was collected throughout a 48-hour "control" period and a second urine collection was made during the subsequent 48-hour "treatment" period. During a treatment period each guinea pig was injected subcutaneously with ACTH which had been suspended in 16% gelatin. Each animal received its total dose in 3 equal portions administered at hours, 0, 12, and 24 of the treatment period. Following the second 48-hour urine collection the guinea pigs were permitted a two-week period without treatment in order to avoid progressive enhancement of adrenal responsiveness to subsequent injections of ACTH.

At bi-weekly intervals the treatments were rotated so that over a period of 12 weeks each group received each of 3 dose levels of both of the two ACTH preparations being compared. (In some assays only two dose levels were used.) The dosage ranges were selected empirically as in the human assays.

## PILOT STUDIES

*Human Assay:*

**17-***Hydroxycorticoids as an index of ACTH activity:* The administration of ACTH to a subject with normal adrenal glands has a variety of physiological sequelae. Three of these have been used most generally in attempts to assay ACTH clinically: the decrease in circulating eosinophils, the increase in urinary 17-ketosteroids and the increase in urinary 17-hydroxycorticoids. Generally there is good correlation among these indices, and they usually go hand in hand with the therapeutic action of ACTH as well. Of these indices of ACTH activity we have found the increase in urinary 17-hydroxycorticoids to be most serviceable since it appears to be a sensitive index of adrenocortical activity over a very wide range. By way of comparison, 17-ketosteroid excretion is less sensitive as an index, and the eosinophil count has value as an index over a relatively narrow range.

*Collection period:* A number of vehicles have been introduced to retard the absorption of ACTH from the site of its injection. At the time this study was carried out the vehicle most commonly used was 16% gelatin. ACTH administered in this vehicle leads to an increased excretion of 17-hydroxycorticoids which usually lasts from 12 to 16 hours. In order to permit the ACTH to exert its full activity during the period of the assay, urine collections were routinely carried out for 24 hours from the time the material was administered.

Control values of 17-Hydroxycorticoids: As the determination was carried out in this laboratory control values of 17-hydroxycorticoids were $9.5 \pm 4$ (2s.d.) mg. per day for

adult males and 6.5 ±2.5 mg. for adult females. A given individual may show considerable variation within this range from day to day. The range of these control values is sufficiently narrow and the absolute magnitude of these values is so small when compared with values obtained during treatment with ACTH that, for practical purposes, the control values may be neglected in carrying out assays. No subjects were employed during periods of extraordinary "stresses" such as those attendant upon surgical procedures, starvation, acute infections, etc.

*Individual differences in responsiveness to ACTH: basis of "cross-over" design:* It was found that among the experimental group of endocrinologically normal subjects there were considerable individual differences in responsiveness to ACTH. These individual differences usually remained relatively constant throughout an assay; that is, subjects who were exceptionally responsive to one dose of a given preparation of ACTH were also relatively responsive to other doses and to other preparations of ACTH. Although these differences in responsiveness gave rise to rather wide scattering of responses to a given dose of ACTH, the effect of this scatter could be eliminated by use of a "cross-over" design whereby each subject received each dose of both the "standard" and "unknown" materials. Such a design has been utilized in the present study.

### *Guinea Pig Assay*

*The Guinea Pig as an Assay Animal:* The fact that the chief adrenal excretory products of both man and the guinea pig are 17-hydroxycorticoids made the guinea pig especially suitable as a laboratory animal for this study, because the same chemical procedure for determining responses to ACTH could be applied to both species.

Within the range of 300 to 500 grams no significant degree of correlation has been found between weight of the animal and responsiveness to ACTH. Zondek and Burstein (1952) have reported cyclical variations in the excretion of formaldehydogenic steroids by female guinea pigs, correlated with estrus. Without exploring this phenomenon further we have carried out our assays in male guinea pigs exclusively.

*17-Hydroxycorticoids as an index of adrenal cortical activation:* It was found in this laboratory that with either chloroform or butanol it was possible to extract from the urine of guinea pigs quantities of "Porter-Silber chromogens" equivalent to from 150 to 400 micrograms of hydrocortisone per adult animal per day. Recent work by Burstein and Dorfman (1954) indicates that practically all of the 17-hydroxycorticoids to be found in guinea pig urine are present in the form of "free" hydrocortisone and tetrahydrocortisone. Since the butanol extract gives a high blank due to non-corticoid chromogenic material, the use of chloroform extracts would appear advantageous. This stands in contrast to the corticoids in human urine, the major portion of which are conjugated with glucuronic acid, (Baggett, Kinsella and Doisy 1953). Of importance in the present study was the finding that the quantity of these corticoids in the urine was a function of the logarithm of the dose of ACTH, over at least a four-fold dosage range.

*Individual animals vs. groups:* Early in our experience it was recognized that a large number of individual animals would be required to establish satisfactorily narrow confidence limits of an estimate of potency. Since the number of corticoid analyses which could be carried out represented a limiting factor in the economy of this assay, it was decided to increase the number of animals but to use the pooled urine of eight guinea pigs receiving identical treatment as a specimen for each *steroid determination.* As a result of this pooling of specimens no increase in the number of steroid determinations was necessary to arrive at satisfactory confidence limits. This end was achieved despite the fact that "lambda" (per animal) remained relatively large when compared with that obtained by Sayers, Sayers, and Woodbury (1948).

*Stress as an interfering factor:* It has been emphasized by Sayers, Sayers and Woodbury (1948) that non-hypophysectomized rats are unsuitable for use in the "intravenous-ascorbic acid depletion" type of assay because the stress of the assay procedure induces endogenous adrenocorticotropic activity. Although the conditions of the "subcutaneous-steroidogenic" assay are not obviously stressful, it is necessary to bear in mind the possible interference which might be introduced by either recognized or unrecognized stressing factors. For this reason an experiment was carried out in which, instead of ACTH, guinea pigs were subjected to (a) starvation for 48 hours, (b) pitressin, 15 pressor units per animal intraperitoneally, and (c) Piromen,[3] 30 micrograms per animal intraperitoneally. The results when compared with those obtained during treatment with ACTH or during treatment with 16% gelatin alone, indicated that although these particular "stressors" induced perceptible increases in 17-hydroxycorticoid excretion, these increases were less than those induced by the smallest doses of ACTH which were employed in the assays herein reported.

## COMPARISON OF PURIFIED ACTH PREPARATIONS

Results of a series of assays indicate, on the one hand, that the steroidogenic effectiveness of ACTH administered extravascularly cannot be predicted on the basis of the official assay alone. On the other hand they indicate close agreement between the steroidogenic responses of man and the steroidogenic responses of guinea pigs when ACTH is administered extravascularly.

*Preparation #16 vs. #284:* This comparison was carried out in six healthy human subjects. The results indicated that one USP Unit of preparation #16 was only one-eighth as effective as #284 when both preparations were administered intramuscularly (Table 1).

[3] Piromen, a purified bacterial polysaccharide, was generously provided by Travenol Laboratories of Morton Grove, Illinois.

TABLE 1. ASSAY OF ACTH IN HUMAN SUBJECTS

| ACTH | #284a | | | #284b | | | #16 | | |
|---|---|---|---|---|---|---|---|---|---|
| Dose U.S.P. units | 5 | 10 | 20 | 5 | 10 | 20 | 20 | 40 | 80 |
| Subject | *24-Hour Urinary 17-Hydroxycorticoids mg.* | | | | | | | | |
| R. | 29 | 37 | 41 | 19 | 33 | 39 | 21 | 29 | 39 |
| H. | 60 | 83 | 103 | 59 | 62 | 72 | 30 | 42 | 81 |
| M. | 15 | 30 | 40 | | | | 12 | 23 | 28 |
| Fa. | 28 | 35 | 42 | 23 | 37 | 48 | 12 | 23 | 30 |
| Ba. | 23 | 27 | 31 | 23 | 28 | 33 | 18 | 25 | 30 |
| Bi. | 27 | 34 | 70 | 43 | 62 | 73 | 28 | 35 | 38 |
| Z. | 35 | 39 | 63 | 21 | 32 | 46 | | | |

All material administered in 16% gelatin vehicle.

$$\frac{\text{Potency \#284b (U.S.P. units)}}{\text{Potency \#284a (U.S.P. units)}} = 0.84$$

95% confidence limits = 0.33–1.69

$$\frac{\text{Potency \#16 (U.S.P. units)}}{\text{Potency \#284 (U.S.P. units)}} = 0.121$$

95% confidence limits = 0.093–0.186

*Replication of Assay of #284:* The assay of #284 was replicated in six human subjects as an empirical check on the reliability of the assay method (Table 1). There was no significant difference between the results of the two assays.

*Preparation #198 vs. #284:* This comparison was carried out in eight human subjects (Table 2) as well as in guinea pigs (Table 3). In both species #198 had slightly greater apparent activity than #284. The difference was not significant at the 5% level. The agreement between the results of this assay in guinea pigs and the corresponding assay in human subjects is obvious.

TABLE 2. ASSAY AND COMPUTATION OF POTENCY OF ACTH IN HUMAN SUBJECTS

| Subject | #284 Dose U.S.P. units 5 U | 10 U | 20 U | #198 Dose U.S.P. units 5 U | 10 U | 20 U | ΔM Material difference | ΔD Dose difference |
|---|---|---|---|---|---|---|---|---|
| | *17-Hydroxycorticoid excretion (mg. per day)* | | | | | | | |
| N | 29 | 52 | 65 | 35 | 41 | 60 | −10 | 61 |
| BU | 34 | 42 | 45 | 39 | 50 | 66 | +34 | 38 |
| W | 38 | 43 | 52 | 39 | 43 | 66 | +15 | 41 |
| G | 23 | 30 | 37 | 21 | 24 | 44 | −1 | 37 |
| Fo | 22 | 37 | 48 | 42 | 48 | 55 | +38 | 39 |
| I | 19 | 23 | 36 | 26 | 32 | 36 | +16 | 27 |
| U | 50 | 75 | 95 | 42 | 67 | 84 | −27 | 87 |
| By | 31 | 48 | 84 | 54 | 60 | 72 | +23 | 71 |
| | | | | | | Total | +88 | 401 |

$$\text{Log potency} = \frac{\sum \Delta M/3}{\sum \Delta D/2} \times .602 = .0880$$

$$\text{Relative potency of } \frac{\text{\#198 (U.S.P. Units)}}{\text{\#284 (U.S.P. Units)}} = \text{antilog } .0880 = 1.225$$

95% confidence limits = 0.89–2.10

TABLE 3. ASSAY OF ACTH IN GUINEA PIGS
Preparation #284 vs. Preparation #198

| Group | | Week | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 3 | 5 | 7 | 9 | 11 |
| 1 | Material, dose | U, 1.0 | U, 0.5 | U, 2.0 | S, 1.0 | S, 0.5 | S, 2.0 |
| | Control | 280 | 260 | 250 | 330 | 230 | 330 |
| | Response | 710 | 490 | 720 | 860 | 440 | 920 |
| 2 | Material, dose | U, 0.5 | U, 2.0 | U, 1.0 | S, 0.5 | S, 2.0 | S, 1.0 |
| | Control | 300 | 220 | 260 | 360 | 240 | 290 |
| | Response | 480 | 700 | 520 | 560 | 800 | 670 |
| 3 | Material, dose | U, 2.0 | U, 1.0 | U, 0.5 | S, 2.0 | S, 1.0 | S, 0.5 |
| | Control | 310 | 250 | 290 | 300 | 270 | 280 |
| | Response | 950 | 500 | 480 | 930 | 580 | 460 |
| 4 | Material, dose | S, 1.0 | S, 0.5 | S, 2.0 | U, 1.0 | U, 0.5 | U, 2.0 |
| | Control | 350 | 280 | 340 | 350 | 250 | 320 |
| | Response | 860 | 470 | 900 | 810 | 420 | 990 |
| 5 | Material, dose | S, 2.0 | S, 1.0 | S, 0.5 | U, 2.0 | U, 1.0 | U, 0.5 |
| | Control | 330 | 280 | 330 | 370 | 330 | 270 |
| | Response | 940 | 630 | 660 | 1060 | 820 | 410 |
| 6 | Material, dose | S, 0.5 | S, 2.0 | S, 1.0 | U, 0.5 | U, 2.0 | U, 1.0 |
| | Control | 380 | 210 | 310 | 410 | 340 | 310 |
| | Response | 620 | 840 | 740 | 800 | 940 | 470 |

Materials: U = ACTH preparation #284 } in 16% gelatin.
S = ACTH preparation #198 }
Dosage expressed in U.S.P. units per animal, total dose.
"Control" and "Response" values represent micrograms of urinary 17-hydroxycorticoids expressed as Compound F, per animal per day.

*Preparation #K53906 vs. #198:* This comparison was carried out in guinea pigs (Table 4) and in one normal human subject (Table 5). The assay in guinea pigs indicated that, unit for unit, #198 had fourteen times the steroidogenic potency of #K53906 when both were administered subcutaneously. There was a corresponding fifteen-fold discrepancy in the human assay. While the number of test subjects was too limited to permit a precise estimate of the relative potencies, the results leave no doubt that #198 was many times more potent than #K53906 in either man or guinea pigs.

## COMPARISON OF DIFFERENT VEHICLES

### *Human and Guinea Pig Assay:*

Not only is it possible to compare the steroidogenic potencies of various ACTH preparations using a common vehicle, but by using the same ACTH preparation in different vehicles, one may evaluate their clinical usefulness. This principle is illustrated in Tables 4 and 6. In guinea pigs as well as in human subjects preparation #198 when administered in an aqueous vehicle was only about $\frac{1}{4}$ as effective as the same preparation administered

TABLE 4. ASSAYS OF THE STEROIDOGENIC EFFECTIVENESS OF TWO ACTH PREPARATIONS IN GUINEA PIGS: THREE DIFFERENT SUBCUTANEOUS VEHICLES

| Group | | Week | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 6 | 8 | 10 | 12 | 14 | 16 | 18 | 20 |
| 7 | Material, dose | S, 1.0 | S, 2.0 | S, 0.5 | W, 4.0 | W, 1.0 | S, 1.0 | S, 2.0 | OPG, 0.33 | OPG, 0.67 | S, 0.5 |
| | Control | 500 | 250 | 340 | 310 | 320 | 240 | 230 | 300 | 230 | 340 |
| | Response | 1150 | 690 | 610 | 700 | 450 | 550 | 660 | 700 | 690 | 540 |
| 8 | Material, dose | S, 2.0 | S, 0.5 | S, 1.0 | W, 1.0 | W, 2.0 | S, 2.0 | S, 0.5 | OPG, 0.67 | OPG, 0.17 | S, 1.0 |
| | Control | 450 | 290 | 360 | 200 | 340 | 310 | 420 | 360 | 190 | 390 |
| | Response | 1530 | 470 | 840 | 310 | 610 | 910 | 700 | 1060 | 290 | 880 |
| 9 | Material, dose | S, 0.5 | S, 1.0 | S, 2.0 | W, 2.0 | W, 4.0 | S, 0.5 | S, 1.0 | OPG, 0.17 | OPG, 0.33 | S, 2.0 |
| | Control | 430 | 260 | 350 | 300 | 360 | 250 | 420 | 250 | 190 | 400 |
| | Response | 620 | 600 | 960 | 500 | 870 | 530 | 880 | 430 | 490 | 1280 |
| 10 | Material, dose | S, 1.0 | S, 1.0 | S, 0.5 | S, 2.0 | S, 1.0 | K, 6.0 | K, 24.0 | S, 0.5 | S, 2.0 | K, 12.0 |
| | Control | 500 | 350 | 510 | 400 | 330 | 250 | 400 | 260 | 240 | 350 |
| | Response | 1200 | 830 | 810 | 1170 | 800 | 450 | 1040 | 480 | 660 | 700 |
| 11 | Material, dose | S, 0.5 | S, 2.0 | S, 1.0 | S, 0.5 | S, 2.0 | K, 12.0 | K, 6.0 | S, 1.0 | S, 0.5 | K, 24.0 |
| | Control | 510 | 200 | 370 | 360 | 380 | 240 | 390 | 280 | 200 | 380 |
| | Response | 750 | 590 | 820 | 540 | 1120 | 510 | 600 | 600 | 340 | 1160 |
| 12 | Material, dose | S, 2.0 | S, 0.5 | S, 2.0 | S, 1.0 | S, 0.5 | K, 24.0 | K, 12.0 | S, 2.0 | S, 1.0 | K, 6.0 |
| | Control | 370 | 340 | 380 | 380 | 390 | 260 | 380 | 340 | 220 | 270 |
| | Response | 970 | 580 | 1220 | 890 | 750 | 1000 | 750 | 1100 | 530 | 320 |

Materials: S—ACTH preparation #198 in 16% gelatin.
W—ACTH preparation #198 in water.
OPG—ACTH preparation #198 in 37% oxypolygelatin.
K—ACTH preparation #K53906 in 16% gelatin.

Dosage expressed as U.S.P. units per animal, total dose.
"Control" and "Response" values represent micrograms of urinary 17-hydroxycorticoids expressed as Compound F per animal per day.

$$\frac{\text{Potency K53906 (U.S.P. units)}}{\text{Potency \#198 (U.S.P. units)}} = .078$$

$$\frac{\text{Potency \#198 in OPG}}{\text{Potency \#198 in 16\% gelatin}} = 2.85$$

$$\frac{\text{Potency \#198 in water (U.S.P. units)}}{\text{Potency \#198 in 16\% gelatin (U.S.P. units)}} = 0.22$$

in 16% gelatin, which in turn was only $\frac{1}{3}$ to $\frac{1}{4}$ as effective as when administered in 37% oxypolygelatin. Here again the agreement between the results of the subcutaneous-steroidogenic assay in the guinea pig and the intramuscular-steroidogenic assay in man is to be noted.

## STATISTICAL ANALYSIS

### *Human Subjects*

In the present assay, standard and unknown were tested in the same subjects. The statistical analysis must therefore allow for the possible reduction in error that such an experimental arrangement makes possible. The method followed is illustrated in Table 2. Eight subjects were tested at each of three dose levels equally spaced logarithmically for each of two ACTH preparations. For each subject we have computed what we call the "material difference," denoted by $\Delta M$ and the "dose difference" denoted by $\Delta D$.

TABLE 5. ASSAYS OF THE STEROIDOGENIC EFFECTIVENESS OF TWO ACTH PREPARATIONS IN 16% GELATIN IN A HUMAN SUBJECT (L)

| Day of study | ACTH | Dose (U.S.P. units) | Response (Urinary 17-hydroxy-) (corticoids mg. per day) |
|---|---|---|---|
| 1 | #198 | 5 | 31 |
| 4 | #198 | 10 | 44 |
| 7 | #198 | 20 | 59 |
| 10 | K53906 | 40 | 20 |
| 13 | K53906 | 80 | 28 |
| 16 | K53906 | 160 | 47 |
| 46 | #198 | 5 | 29 |
| 49 | #198 | 10 | 48 |
| 52 | #198 | 20 | 50 |

$$\frac{\text{Potency K53906 (U.S.P. Units)}}{\text{Potency \#198 (U.S.P. Units)}} = .065$$

TABLE 6. THE EFFECT ON STEROIDOGENESIS OF DIFFERENT VEHICLES USED INTRAMUSCULARLY IN A NORMAL HUMAN SUBJECT (L) GIVEN LOT #198

| Day of study | Vehicle | Dose U.S.P. units | Response urinary 17-hydroxy-corticoid mg. per day |
|---|---|---|---|
| 1 | 16% Gelatin | 5 | 31 |
| 4 | | 10 | 44 |
| 7 | | 20 | 59 |
| 10, 13, 16 | — | — | — |
| 19 | Water | 10 | 17 |
| 21 | | 20 | 31 |
| 23 | | 40 | 37 |
| 25 | 37% Oxypolygelatin | 5 | 53 |
| 32 | | 10 | 75 |
| 39 | | 20 | 103 |
| 46 | 16% Gelatin | 5 | 29 |
| 49 | | 10 | 48 |
| 52 | | 20 | 56 |

$$\frac{\text{Potency \#198 in water (U.S.P. Units)}}{\text{Potency \#198 in 16\% Gelatin (U.S.P. Units)}} = 0.24$$

$$\frac{\text{Potency \#198 in OPG (U.S.P. Units)}}{\text{Potency \#198 in 16\% Gelatin (U.S.P. Units)}} = 4.26$$

TABLE 7. ESTIMATED VALUES OF s, b AND $\lambda$, THREE ASSAYS IN HUMAN SUBJECTS

| Assay | s | b | $\lambda$ |
|---|---|---|---|
| 198–284 | 9.1 | 41.6 | 0.22 |
| 284–16 | 12.4 | 37.4 | 0.33 |
| 284[a]–284[b] | 14.4 | 38.9 | 0.37 |
| Pooled | 11.8 | 39.5 | 0.30 |

Thus for subject "*N*"

$$\Delta M = (35+41+60)-(29+52+65) = -10$$
$$\Delta D = (65-29)+(60-35) = 61$$

The estimate of the common slope (per fourfold dilution) is then simply the average value of $\Delta D/2$. Similarly the estimate of log potency is

$$\left[\frac{\text{Total } (\Delta M)}{3} \div \frac{\text{Total } (\Delta D)}{2}\right] \times \log 4.$$

When the dosage levels of standard differ from those of unknown, as for example in Table 3, an appropriate correction must be made. As indicated at the foot of the table, preparation #198 is then estimated to be 1.23 times as potent as preparation #284.

To obtain confidence limits for this estimate of potency, we note that log potency is defined as the ratio of two quantities. The confidence limits may be obtained from the expression of Fieller (1944) for confidence limits for the ratio of two normally distributed variables, whose variances and covariances can be estimated from the individual $\Delta M$ and $\Delta D$. On this basis the 95% confidence limits for the estimate of potency are 0.89–2.10.

This procedure has been followed in calculating confidence limits for each potency estimate presented in this paper. However, a quick approximation is given by the expression

$$\frac{.401}{1-g}\left\{\frac{\text{Tot. } (\Delta M)}{\text{Tot. } (\Delta D)} \pm \frac{t \times \text{Range } (\Delta M)}{\text{Tot. } (\Delta D)}\sqrt{1+\left[\frac{\text{Tot. } (\Delta M)}{\text{Tot. } (\Delta D)}\right]^2}\right\} + \log R$$

Where

t = Student's t for the chosen probability level and degrees of freedom and equals 2.365 for 95% confidence limits for the data in Table 2

Range $(\Delta M)$ = Largest $(\Delta M)$ − Smallest $(\Delta M)$
= 38 − (−27) for Table 2

$.401 = \frac{1}{3} \div \frac{1}{2} \times \log 4$

$$g = \left[\frac{t \text{ Range } (\Delta D)}{\text{Total } (\Delta D)}\right]^2$$

= .126 for Table 2

$R$ = ratio of dosage of unknown to standard
= 20/20 for Table 2
= 80/20 for Table 1 (#16 vs. #284).

The limits of the log potency so calculated are −.079 to .281. The antilogs 0.83 to 1.91 provide a useful approximation to the exact limits 0.89 to 2.10. The approximation should not be used when the number of observations exceeds 15.

It is of some interest to estimate the value of λ, the index of inherent precision, defined as s/b, where s is the standard deviation of a single observation and b is the slope per tenfold dilution. Thus, in Table 2, to estimate the effective s for this experimental arrangement we note that the variance of the $\Delta M$'s ,496, is 6 $s^2$, so that s is 9.1. Since b is 41.6, λ is 0.22. In Table 7 we summarize the estimates of s, b and λ obtained from three different assays carried out in human subjects. The pooled estimate of λ obtained from all three experiments is 0.30.

How much larger would λ be if each subject were used only once, rather than six times as in the present assay? The slope would, of course, remain unchanged, but because

TABLE 8. COMPUTATION OF POTENCY IN GUINEA PIG ASSAY

| Guinea pig group | #284 | | | #198 | | | Material difference | Dose difference |
|---|---|---|---|---|---|---|---|---|
| | 0.5 U | 1.0 U | 2.0 U | 0.5 U | 1.0 U | 2.0 U | | |
| | *Increase over control value in 17-hydroxycorticoid excretion (micrograms per animal per day)* | | | | | | | |
| 1 | 230 | 430 | 470 | 210 | 530 | 620 | +230 | 650 |
| 2 | 180 | 260 | 480 | 200 | 380 | 560 | +220 | 660 |
| 3 | 190 | 250 | 640 | 180 | 310 | 630 | +40 | 900 |
| 4 | 170 | 460 | 670 | 190 | 510 | 560 | −40 | 870 |
| 5 | 140 | 490 | 690 | 330 | 350 | 610 | −30 | 830 |
| 6 | 390 | 160 | 600 | 240 | 430 | 630 | +150 | 600 |
| | | | | | | Total | 570 | 4510 |

$$\log \text{potency} = \frac{\sum \Delta M/3}{\sum \Delta D/2} \times .602 = .0506$$

$$\text{relative potency of } \frac{\#198 \text{ (U.S.P. Unit)}}{\#284 \text{ (U.S.P. Unit)}} = \text{antilog. } .0506 = 1.124$$

$$95\% \text{ confidence limits} = 0.94\text{–}1.34$$

of differences in the level of responsiveness among individuals, s would increase. We can estimate s for this experimental arrangement from the variation in the quantity of excreted steroids between individuals within each dose level. For the assay shown in Table 2 the value of s so estimated is 15.0, while a pooling of all three human assays gives an estimate of s of 16.9 and a $\lambda$ of 0.43. The effect of employing the same individuals repeatedly has thus been to decrease $\lambda$ from 0.43 to 0.30 and to provide equally accurate estimates of potency with half $(.30^2)/.43^2$ as many observations.

*Guinea Pigs*

We have followed identical procedures in estimating potency and the required confidence limits for the guinea pig assay. An illustrative computation on six groups of guinea pigs, each group containing 8 animals, is shown in Table 8. Preparation #198 is estimated to be 1.12 times as potent as #284. and 95% confidence limits for the relative potency are 0.92 to 1.39. Estimates of s, b and $\lambda$ obtained as in the human assay are 49.6, 624.3 and 0.079. Each group of guinea pigs includes 8 animals, however, so that the $\lambda$ obtained must be multiplied by $\sqrt{8}$ to obtain an estimate of variation among individual guinea pigs. Thus s, per animal becomes 140.3 and $\lambda$ 0.22. In Table 9 we summarize the estimates of s, b and $\lambda$ obtained from the four assays of unknowns using guinea pigs. The pooled estimate of $\lambda$ is 0.31, or the same as in the human assay. Thus the responses of one guinea pig (with control value) and of one human subject (without control value) furnish almost equal amounts of information in the present assay.

TABLE 9. ESTIMATED VALUES OF s, b AND $\lambda$, FOUR ASSAYS USING GUINEA PIGS

| Assay | s | b | $\lambda$ |
|---|---|---|---|
| 198–284 | 140 | 624 | 0.22 |
| W—198 | 76 | 695 | 0.11 |
| OPG—198 | 318 | 490 | 0.65 |
| K—198 | 239 | 803 | 0.30 |
| Pooled | 197 | 645 | 0.31 |

## DISCUSSION

Almost from the time that ACTH was first distributed for general use in clinical medicine it has been apparent that some modification of the official method of assaying potency of various preparations was desirable. Numerous reports have appeared citing discrepancies between the labelled potency of ACTH and the clinical activities of various preparations when administered extravascularly. (Brown and Greenblatt, 1952; Wolfson and Robinson, 1952; Mandel, *et al.*, 1951; Renold, *et al.*, 1952; Raben *et al.*, 1952; Segaloff, *et al.*, 1952; Thompson and Fisher, 1953; and Ogryzlow and Gornall, 1953).

There are three major sources of error in estimating the therapeutic effectiveness of ACTH: first, variability in the amount of corticoid required to control a particular patient's disease process; second, individual differences in adrenal responsiveness to ACTH; and third, variability in the degree of local tissue inactivation of different "lots" of ACTH. It is this third source of error which might be eliminated by some variety of "extravascular-steroidogenic" assay. A number of approaches to this problem have been proposed. For example, Fisher and Thompson (1952) have assayed ACTH in rats in terms of adrenal ascorbic acid depletion 3 hours after the subcutaneous injection of the test material. Speirs (1953) has employed the eosinopenic response to subcutaneously-injected ACTH. Wolfson (1953) has developed a complex assay scheme based upon therapeutic, hematologic, and steroidogenic responses in human subjects.

The "extravascular-steroidogenic" assay method described above appears to have the dual advantage of being relatively simple yet quite direct, in that it yields information in terms of therapeutically active steroids of adrenal origin and their immediate derivatives. The close agreement between the results obtained employing guinea pigs and those obtained in human subjects offers hope that assays in the former have potential clinical relevance.

Assays carried out in animals with intact pituitary glands may be affected by non-specific stress. Results of our studies indicate that this source of error is not serious when one employs purified ACTH preparations in the relatively large doses cited above. The method outlined would not be applicable to isolation work where very small amounts of impure material must be evaluated.

It is to be expected that the precision and simplicity of the present method might be improved by (a) use of a single injection rather than three injections for each test, and (b) substitution of a simpler steroid technique for the present Porter-Silber method. The latter can be achieved, for example, by adoption of a "17-Ketogenic" steroid (Norymberski Stubbs, and West, 1953) method which has already shown promise in preliminary studies. (Island and Forsham, 1954).

SUMMARY

ACTH is susceptible to inactivation by local tissues, the degree of which varies from one preparation to another. The established assay methods which use the intravenous route of administration do not enable one to predict the degree of inactivation which might occur when the hormone is administered extravascularly. Discrepancies between the labelled potency and the clinical effectiveness of ACTH may result.

A new method of assaying ACTH has been described, in which ACTH is injected subcutaneously into guinea pigs and its effectiveness is assessed in terms of urinary steroids of adrenal origin (17-hydroxycorticoids). With this method it is possible to evaluate not only the potency of ACTH but the usefulness of various ACTH vehicles as well.

Consistent agreement has been found between the results of this assay method in guinea pigs and the results of similar assays performed in normal human subjects.

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# THE RESPONSE OF HYPOPHYSECTOMIZED MALE KILLIFISH TO PROLONGED TREATMENT WITH SMALL DOSES OF THYROTROPIN[1]

GRACE E. PICKFORD

*Bingham Oceanographic Laboratory, Yale University*

PREVIOUS investigations (Pickford, 1953b; 1954) have shown that highly purified beef growth hormone preparations contained sufficient thyrotropin to maintain the thyroids of hypophysectomized killifish at a normal level of activity, although there was no evidence of hyper-stimulation. According to Dr. A. E. Wilhelmi, who supplied the growth hormone preparations, the presence of thyrotropin could not be detected by the rat test and it was believed that a contamination of the order of 1% was the maximum that could be expected. The following experiment was undertaken at his suggestion to determine the minimum amount of a standard preparation of thyrotropin that would be sufficient to account for the results noted above. Since the growth hormone was administered at a dosage level of 10 μg. per gram weight thrice weekly, the treatment with thyrotropin was calculated to straddle the 1% level of contamination, at 1 μg. 0.1 μg. and 0.01 μg. per gm. weight. The standard preparation employed was Armour's thyrotropin 2R3. Controls received 0.6% NaCl. The conditions of the experiment conformed to those used previously (Pickford, 1954) and procedures were identical, except that the experiment was terminated at the end of one month. In view of the close association of thyrotropin with the exophthalmos producing factor, and of the use of killifish for the assay of the latter (Albert, 1945; Dobyns and Steelman, 1953), measurements of interocular width were made at weekly intervals. At autopsy attention was paid to the color of the blood, liver and testes weights, and presence of renal calculi, in order to ascertain whether treatment with thyrotropin would alleviate any of the collateral effects of hypophysectomy in killifish (Pickford, 1953a, b; 1954).

I should like to thank my assistants, Miss Harriet A. Chambers and Mr. Nicol Cooper (Harvard '56) for their help in the investigation.

## RESULTS

At the highest dosage level, 1 μg. per gm. weight, the thyroids were strongly stimulated (Table 1); the average cell height (7.6 μ) is well above

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TABLE 1. CONDITION OF FISH AT AUTOPSY: HEMOGLOBIN, EXOPHTHALMOS, AND THYROID

| Treatment | No. of fish | Hemoglobin | | Exophthalmos | | Average thyroid cell ht | | Average no. cells 12$\mu$ and over, % | Azan red colloid follicles % |
|---|---|---|---|---|---|---|---|---|---|
| | | % | s | % | s | % | s | | |
| TSH, 1 $\mu$g./gm. | 6 | 76 | ±6 | 3.47 | ±2.61 | 7.6 | ±1.9 | 8.5[1] | 54 |
| TSH, 0.1 $\mu$g./gm. | 6 | 80 | ±7 | 1.46 | ±2.15 | 4.3 | ±0.7 | 0 | 53[2] |
| TSH, 0.01 $\mu$g./gm. | 5 | 66 | ±6 | 0.70 | ±1.65 | 3.6 | ±0.1 | 0 | 77 |
| 0.6% NaCl | 5 | 68 | ±6 | −0.75 | ±0.79 | 3.8 | ±0.2 | 0 | 57 |

[1] Range = 1 to 18%.
[2] One fish in this group had only 2 Azan red (resting) follicles, the average thyroid epithelial height was 5.07$\mu$.

normal values for unoperated aquarium fish (5.0-5.4$\mu$; Pickford 1953a) and well above the levels observed in the growth hormone experiments (Pickford, 1953b; 1954). At the medium dosage level, 0.1 $\mu$g. per gm. weight, the average cell height is slightly greater than that of the saline controls but the difference is not statistically significant (Table 3). Nevertheless, 2 of the 6 fish in this group had thyroids that were definitely normal in character, with average follicle cell heights of 5.07 and 5.26 $\mu$ respectively. Clearly this dosage level is slightly below, but very close to the minimum that would be required to maintain the thyroids of all the hypophysectomized fish at a normal level.

The development of exophthalmos closely parallels the stimulation of the thyroid (Table 1). At the highest dosage level an increase in interocular width of the order of 3.5% was established at the end of the first week and maintained for the duration of the experiment. At the medium dosage level there was a small increase in interocular width that was suggestive but hardly statistically significant (Table 3). As with the thyroid, it is evident that a dosage level of 0.1 $\mu$g. per gram weight is just barely sufficient to elicit the response.

Among collateral effects the alleviation of anemia due to hypophysectomy is of particular interest (Table 1). Fish receiving thyrotropin both at 1 and 0.1 $\mu$g. per gm. weight showed normal hemoglobin levels and the differences between these fish and the controls were definitely significant (Table 3). It has been claimed by Kalashnikov and Skadovsky (1940, 1948) that treatment with thyroxin alleviated the anemic condition of captive sturgeons. However, recent investigations of Contropoulos *et al.* (1954) have indicated that the erythropoietic factor of the pituitary is independent of any of the known target organs of the anterior lobe. It is therefore probable that the preparation used in our experiments contained the erythropoietic factor.

It will be seen from Table 2 that treatment with thyrotropin had no significant effect on the slight progressive weight loss of hypophysectomized killifish. On the other hand, at the highest dosage level, there was a small

TABLE 2. CONDITION OF FISH AT AUTOPSY: WEIGHT AND LENGTH CHANGES, GONOSOMATIC INDEX (GSI), HEPATOSOMATIC INDEX (HSI), AND INCIDENCE OF VISIBLE URINARY CONCRETIONS

| Treatment | No. of fish | No. with kidney stones | Average weight change | | Average length change | | Average GSI | | Average HSI | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | % | s | % | s | % | s | % | s |
| TSH, 1 μg./gm. | 6 | 1 | −2.2 | ±3.2 | 0.86 | ±0.56 | 0.48 | ±0.08 | 4.9 | ±1.0 |
| TSH, 0.1 μg./gm. | 6 | 0 | −5.1 | ±7.1 | 0.13 | ±0.48 | 0.37 | ±0.11 | 4.5 | ±0.8 |
| TSH, 0.01 μg./gm. | 5 | 2 | −6.3 | ±3.9 | 0.16 | ±0.34 | 0.28 | ±0.05 | 4.4 | ±0.4 |
| 0.6% NaCl | 5 | 2 | −1.2 | ±5.2 | −0.17 | ±0.71 | 0.29 | ±0.05 | 5.4 | ±1.6 |

but statistically significant length increment at the end of one month (Tables 1 and 3). Further consideration of the data suggests that this merely represents a partial restoration of the length decrement, 1.81 ± 0.86%, which ensued during the two months period of hypophysectomy previous to the start of the experiment. It may thus be attributed to the improved health of the fish consequent to thyroid stimulation, and can hardly be regarded as evidence for the resumption of growth.

At the highest dosage level the testes showed a slight but significant degree of stimulation (Tables 2 and 3). According to Piotrowski, Steelman and Koch (1953) Armour's thyrotropin No. 2R3 contains 6 Collip units of gonadotropin per milligram. Evidently even this small amount of gonadotropin, administered over a prolonged period of time, is sufficient to effect a slight stimulation of the regressed testes of hypophysectomized killifish. The degree of stimulation was not sufficient to elicit the development of nuptial coloration.

Treatment with thyrotropin, even at the 1 μg. per gm. dosage level, had no effect on liver size (Tables 2 and 3), nor was there any alleviation of the incidence of renal lithiasis (Table 2).

## SUMMARY

Hypophysectomized male killifish (Fundulus heteroclitus) were injected thrice weekly at 20° C. for one month with Armour's thyrotropin No. 2R3

TABLE 3. CONDITION OF THE FISH AT AUTOPSY: PROBABILITY LEVELS (IN PER CENT) OF SIGNIFICANCE OF THE DIFFERENCES BETWEEN THE GROUPS RECEIVING THYROTROPHIN AND THE SALINE CONTROLS[1]

| Treatment | Weight | Length | GSI | HSI | Hemoglobin | Exophthalmos | Thyroid cell ht. |
|---|---|---|---|---|---|---|---|
| TSH, 1 μg./gm. | — | 2–5% | 0.1% | — | 2–5% | 0.1–1% | 0.1% |
| TSH, 0.1 μg./gm. | — | (10–20%) | — | — | 1–2% | (5–10%) | (10–20%) |
| TSH, 0.01 μg./gm. | (10–20%) | — | — | — | — | (10–20%) | — |

[1] Probability levels above 20% are not listed (—). Values between 5% and 20%, which are of highly doubtful significance, are enclosed in parentheses.

at dosage levels of 1 μg., 0.1 μg. and 0.01 μg. per gram weight per injection. The highest dosage level caused thyroid stimulation and maintained a low degree of exophthalmos. The medium dosage level was barely sufficient to maintain the thyroids at a normal level and induced a very slight degree of exophthalmos. At both these dosage levels the treatment relieved the anemia due to hypophysectomy. The treatments had no effect on the liver size or incidence of renal lithiasis but, at the highest dosage level, there was a slight stimulation of the testes.

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# FASTING KETOSIS IN THE ADRENALECTOMIZED AND CORTISONE-TREATED RAT

MILDRED G. ENGEL AND FRANK L. ENGEL[1]

*Departments of Medicine and Physiology, Duke University, Durham, North Carolina*

CONCEPTS concerning the role of the adrenal cortex in ketone metabolism have been colored by the universal finding that ketonuria during fasting or after phloridzin poisoning is essentially abolished by adrenalectomy. (Evans, 1936; MacKay and Barnes, 1937; MacKay and Wick, 1939; Samuels and Conant, 1944; Segaloff and Many, 1952). On the other hand when blood ketone levels have been measured in adrenalectomized animals values not grossly different from normal have been found frequently and it has become apparent that failure of renal excretion of ketone bodies is a large factor accounting for the absence of ketonuria after adrenalectomy (Mirsky, 1938; Shipley and Long, 1938; MacKay and Wick, 1939, MacKay *et al.*, 1946; Bahner 1953).

Recently it has been reported from this laboratory (Scott and Engel, 1953) that both cortisone and hydrocortisone depress fasting and cold-stress ketosis in the rat, a result consistent with the report of Kinsell *et al.* (1952) that ACTH and cortisone block fasting ketosis in man. This finding plus observations on the effect of changes in the circulation on fasting ketosis led us to reinvestigate the effect of adrenalectomy on fasting ketosis. It is well known that the circulation of the adrenalectomized rat not maintained with hormone is not up to par. In the normal rat depression of the circulation by a small hemorrhage inhibits fasting ketosis while frank hemorrhagic shock actually lowers blood ketone levels (Engel and Hewson, 1953). It seemed possible that enhanced ketonemia might be a feature of the state of adrenal insufficiency but is obscured by the ketone-lowering effect of the suboptimal circulation of this preparation. Careful search of the literature revealed one report (Thaddea and Kuhn, 1937) indicating that adrenal insufficiency in the cat may in fact be associated with elevated blood ketone levels during fasting and that these may be restored to normal by treatment with adrenal cortical extract.

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In the present study it was found that adrenalectomized rats kept in good condition with DCA and salt did indeed exhibit significantly greater ketonemia during a four day fast than did normal controls or adrenalectomized rats maintained with cortisone and saline, cortisone, DCA and saline or with saline alone. Additional observations on the effect of cortisone on ketosis in normal rats during a 10 day fast are also reported.

## METHODS

Male albino rats of the Vanderbilt strain, weighing approximately 200–250 grams were used. Prior to operation and fasting they were maintained on a diet of commercial chow, supplemented with liver pudding and lettuce twice weekly (Engel, Schiller and Pentz, 1949). The animals were adrenalectomized by the lumbar approach under light nembutal anesthesia. Immediately after operation hormone treatment was begun, the dosages being 0.5 or 1.0 mg. of cortisone acetate as a crystalline suspension in saline (5 mg. cortisone acetate/ml.) and 0.5 mg. of desoxycorticosterone acetate in oil subcutaneously. All animals were kept in individual cages and on the third post-operative day food was withdrawn. Blood samples were taken without anesthesia from the tail at this time and at 24 hour intervals thereafter for 4 days and analyzed for ketone bodies by a modification of the method of Greenberg and Lester (1944, 1948) and for glucose by the method of Somogyi (1945). Only animals that survived the four day fast were included. Rats were tested for the completeness of the adrenalectomy by keeping them on a regular diet without added saline until death. Animals that survived more than 4 weeks were discarded. This long period was used to allow for the disappearance of cortisone from the subcutaneous depots.

## RESULTS

*Effects of cortisone on ketonemia and glycemia during a prolonged fast.* The data of Table 1 supplement those of a previous report (Scott and Engel, 1953) by demonstrating that the suppressive effect of cortisone on fasting ketosis persists for as long as eight days. In general, blood sugar levels were significantly higher in the cortisone treated group. This sustained effect of cortisone is noteworthy in view of other reports indicating that some cortisone effects may begin to decline after 7–8 days of continuous treatment (Winter *et al.*, 1950; Ingle *et al.*, 1953) while other effects such as ketonuria in Ingle's rats force fed a high fat diet, may appear only after 7–8 days of treatment (Ingle, Prestrud and Li, 1951; Ingle, Prestrud and Nezamis, 1951). In our previous report a possible late effect of cortisone was sought for by not beginning the fast until the seventh day of cortisone treatment. Ketosis was effectively inhibited under these experimental conditions as well.

*Effect of adrenalectomy and replacement therapy on fasting ketonemia and glycemia.* Table 2 records the blood sugar and ketone levels of normal and adrenalectomized rats prior to and during a four day fast. Compared to the normal controls the saline-maintained adrenalectomized rats had signifi-

TABLE 1. EFFECT OF CORTISONE ACETATE ON FASTING KETONEMIA AND GLYCEMIA

| Days of fasting | | 0 | 1 | 2 | 3 | 4 |
|---|---|---|---|---|---|---|
| Blood ketones as Mg. acetone per 100 ml. | Control | 1.05±0.42 (5) | 4.38±0.35 (12) | 6.93±0.78 (13) | 6.88±0.19 (12) | 6.01±0.83 (13) |
| | Cortisone acetate 5 mg./day* | 0.78±0.09 (6) | 2.27±0.35 (9) | 2.55±0.51 (9) | 1.99±0.26 (9) | 1.55±0.14 (9) |
| Blood glucose Mg./100 ml. | Control | 109±1.8 (11) | 87±3.0 (11) | 87±2.8 (11) | 96±3.3 (11) | 95±3.0 (10) |
| | Cortisone acetate 5 mg./day* | — | 110±1.8 (3) | 113±5.6 (6) | 110±3.5 (9) | 119±2.6 (9) |

| Days of fasting | | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|
| Blood ketones as Mg. acetone per 100 ml. | Control | 6.34±1.58 (6) | 4.41±0.87 (11) | 5.09±1.24 (7) | 4.35±0.13 (8) | 2.41±0.81 (6) | 3.20±1.40 (7) |
| | Cortisone acetate 5 mg./day* | 2.06±0.31 (9) | 1.47±0.14 (6) | 1.81±0.11 (9) | 1.67±0.21 (8) | 0.97±0.16 (3) | 1.29±0.12 (2) |
| Blood glucose Mg./100 ml. | Control | 93±2.0 (10) | 106±5.3 (10) | 93±3.5 (10) | 97±5.3 (10) | 104±3.1 (8) | 98±2.3 (7) |
| | Cortisone acetate 5 mg./day* | 121±3.3 (8) | 130±2.6 (3) | 130±3.9 (8) | 117±4.8 (9) | — | 113±1.9 (3) |

Data expressed as mean ± standard error. Values underlined are significantly different from corresponding controls.
* Cortisone injections begun 48 hours before beginning the fast.

cantly lower ketone levels when fed and during the first two days of fasting while the DCA treated group had significantly higher levels of ketone bodies during days 2, 3 and 4. Blood sugar levels were significantly lowered in both the saline and DCA group during all the fasting days. Adrenalectomized rats treated with 0.5 mg. cortisone (D) or with 1.0 mg. cortisone and 0.5 mg. DCA (F) exhibited normal blood ketone and sugar values. The ketone levels in general were less than in the DCA group (C) while the blood sugar values were higher. An exception was the significantly high blood ketone on zero day in group F. This is probably an artefact due to the fact that blood samples in this group were taken at 2:00 P.M. instead of 9:00 A.M. as in the other groups and in all likelihood the animals did not eat during the morning. In group E which received 0.5 mg. cortisone and 0.5 mg. DCA the results were rather variable with the ketone levels not being significantly different from either the controls or the DCA group, but closer to the latter. In view of the fact that in group E the blood sugar levels tended to be lower than in the controls and on two days not significantly different from the DCA group it is possible that for some reason the dose of cortisone was not as adequate for maintenance as it was in group D. Analysis of the weight loss during the 4 days of fasting, however, indicated no differences between the normal controls and the three groups receiving cortisone, while the saline and DCA treated groups lost significantly less weight. In order to rule out the possibility that DCA itself might increase fasting ketosis, a series of normal rats were treated with 2.5 mg. DCA daily for 3 days and then fasted for 4 days, the DCA treatment

TABLE 2. FASTING KETOSIS AND GLYCEMIA IN ADRENALECTOMIZED RATS

| Experimental groups | Fasting days | | | | |
|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 |
| | Blood Ketones, mg. Acetone/100 ml. | | | | |
| A. Normal Control | 1.11±0.11 (17) | 4.55±0.30 (17) | 4.90±0.34 (17) | 3.83±0.31 (17) | 3.31±0.41 (17) |
| B. Adrx—Saline | 0.55±0.05 (11) | 3.13±0.15* (11) | 3.64±0.17* (11) | 3.68±0.45* (11) | 3.71±0.69 (10) |
| C. Adrx—0.5 mg. DCA | 1.10±0.24 (6) | 5.23±0.32 (9) | 7.92+0.38 (9) | 8.40+0.61 (9) | 6.01+0.98 (8) |
| D. Adrx—0.5 mg. Cortisone | 0.95±0.11 (11) | 4.15±0.34 (11) | 5.13±0.51* (11) | 4.68±0.81* (10) | 3.79±0.91 (9) |
| E. Adrx—0.5 mg. Cortisone —0.5 mg. DCA | 1.01±0.18 (6) | 5.42±0.43 (6) | 6.60+0.58 (6) | 6.50+1.04 (6) | 4.55±1.62 (5) |
| F. Adrx—1.0 mg. Cortisone —0.5 mg. DCA | 2.13±0.09* (6) | 5.02±0.55 (6) | 6.00±1.21 (6) | 4.19±0.81* (6) | 3.93±0.87 (5) |
| | Blood Sugar—mg./100 ml. | | | | |
| A | 98±3.8 (17) | 72±2.5 (17) | 73±2.9 (13) | 76±3.0 (17) | 79±2.6 (17) |
| B | 105±2.1 (11) | 55±2.1 (11) | 54±2.8 (11) | 51±3.6 (11) | 53±5.0 (10) |
| C | 103±4.3 (6) | *61±2.9* (9) | 52±2.7 (9) | 54±2.9 (9) | 52±2.8 (8) |
| D | 97±2.5 (11) | 69±1.4† (11) | 67±2.7* (11) | 78±2.9* (10) | 73±5.3* (9) |
| E | 93±4.8 (6) | **61±1.4** (6) | 63±2.9† (6) | 70±6.0 (6) | 65±10 (5) |
| F | 104±2.0 (6) | 71±3.3† (6) | 75±5.1* (5) | 80±4.6* (6) | 82±1.9* (5) |

Data expressed as mean ± standard error.
Values underlined $p < .01$ compared to Group A.
Values in italics $p < .02$ compared to Group A.
Values in bold type $p < .05$ compared to Group A.
* $p < .01$ compared to Group C.
† $p < .02$ compared to Group C.

being continued. This treatment had no effect on fasting ketosis or glycemia. The data will not be recorded here.

## DISCUSSION

This study demonstrates that under the proper experimental conditions ketonemia is enhanced during fasting in the adrenalectomized rats, a finding which is consistent with the reverse observation that ocrtisone and hydrocortisone suppress fasting ketosis. In light of the fact that impairment of the circulation by hemorrhage or shock suppresses fasting ketonemia in the normal rat, (Engel and Hewson, 1953) the most likely explanation for the previous failures to demonstrate fasting ketosis after adrenalectomy and for the lesser degree of ketonemia in the saline treated group is that the state of the circulation after adrenalectomy was such as to modify ketone metabolism in a direction opposite to that resulting from adrenal in-

sufficiency. The importance of the metabolic effects of circulatory failure in interpreting studies on the adrenalectomized animal has been emphasized previously (Engel, 1951). Another example of this phenomenon is found in the studies of Russell who reported a high rate of glucose utilization in the saline maintained adrenalectomized eviscerated rat but a normal rate in the DCA maintained adrenalectomized eviscerated rat (Russell, 1943). She attributed the high rate of glucose utilization in the saline group to the failing circulation in this preparation since a similar high rate of glucose utilization could be induced by a mild hemorrhage in the eviscerated rat with intact adrenal glands (Russell, Long and Engel, 1944). The recent observations that adrenergic blocking agents considerably enhance the ability of the adrenalectomized animal to resist stress may be taken as another example of the importance of the state of the vascular system in determining circulatory and metabolic decompensation after adrenalectomy (Goldstein *et al.*, 1952).

The level of ketone bodies in the blood is determined by the balance between ketone production by the liver and utilization by other tissues. In the case of the shocked rat the data suggested that ketone body production by the liver was probably depressed, but the possibility that ketone utilization elsewhere was concomitantly increased was also considered and is currently under investigation (Engel and Hewson, 1953). There are reasons to believe that the same mechanisms are at play in the saline maintained adrenalectomized animals although the data on the subject are contradictory. Lipsett and Moore (1951, 1952) reported decreased endogenous and exogenous ketone production by liver slices from adrenalectomized mice while Bondy and Wilhelmi (1950) found no difference from normal in ketone production by liver slices from adrenalectomized rats, all maintained on saline. Nelson *et al.* (1940) described decreased ketone-body utilization in saline treated fed adrenalectomized rats as measured by the disappearance of β-hydroxybutyrate injected intraperitoneally and interpreted this as indicating a decreased hepatic production of ketone bodies by the liver since blood ketone levels were not found to be increased. MacKay, Wick and Barnum (1946), on the other hand, found that arteriovenous ketone differences were higher than normal in fasted saline treated adrenalectomized rats. Since blood ketone levels were normal and occasionally above normal in their operated rats they believed that there must be an enhanced rate of hepatic ketone body production in the adrenalectomized rat. Since studies comparable to the above have not been performed on DCA maintained rats and the possible role of circulatory failure has not been considered in any of these experiments one must suspend judgment as to their significance in relation to the present observations.

If one accepts the DCA maintained adrenalectomized rat as a preparation in which the circulatory factor has been stabilized it is possible to formulate an hypothesis to account for the observed effects of adrenalectomy and cortisone treatment on fasting ketosis in terms of current concepts of the role of the adrenal cortex in fat metabolism. Welt and Wilhelmi (1950) and Wilhelmi (1953) have shown that the adrenalectomized rat fed a high carbohydrate, fat-free diet exhibits a high rate of lipogenesis from carbohydrate as well as an accelerated rate of fat utilization so that in general this preparation does not accumulate fat despite the high rate of fat synthesis. Conversely, several investigators have presented evidence that ACTH or cortisone treatment inhibits fat synthesis from carbohydrate while at the same time there may be a net accretion of fat in the carcass, suggesting impaired fat catabolism (Welt and Wilhelmi, 1950; Brady, Lukens and Gurin, 1951; Balmain, Folley and Glasscock, 1952; Wilhelmi, 1953; Breibart and Engel, 1954). According to current concepts the production of ketone bodies by the liver may be looked upon as an alternative pathway in the metabolism of acetate, occuring whenever acetate (acetyl CoA) exists in excess and either cannot be oxidized via the citric acid cycle because of a paucity of oxalacetate from carbohydrate or protein catabolism or cannot be converted to fat (Engel, 1953). In the fasted normal animal ketosis is presumably a reflection of an acetate excess due to the increased production of this substance from fat catabolism coupled with a decreased ability to synthesize fat from acetate and to provide sufficient oxalacetate from preformed carbohydrate or from enhanced tissue protein catabolism to allow full oxidation of the acetate in the liver via the citric acid cycle. One might speculate that following adrenalectomy continued lipogenesis from carbohydrate and acetate might persist in contrast to the normal animal in which this process comes to a standstill. In terms of acetate utilization, however, presumably this process is overbalanced by enhanced fat catabolism. The latter added to the combination of decreased carbohydrate stores and diminished protein catabolism to serve as sources of oxalacetate then sets the stage for the ready development of ketosis. Conversely, while cortisone treatment of the normal animal favors ketosis by inhibiting fat synthesis, this effect may be more than overbalanced by a greater decrease in fat catabolism, by an increase in carbohydrate production by the liver (Welt *et al.*, 1952, Renold and Hastings, 1953) and an increase in regeneration of citric acid cycle precursors from protein catabolism, thus inhibiting ketosis during fasting. Thus, the observed effects of cortisone and adrenalectomy on fasting ketosis can be fit readily into current concepts of ketosis and of the mode of action of the adrenal cortex in intermediary metabolism.

SUMMARY

Cortisone treatment inhibits ketonemia and elevates the blood sugar during a ten day fast in normal rats.

Adrenalectomized rats maintained with saline alone exhibit significantly less ketonemia and have lower blood sugars during a four day fast than do normal or cortisone maintained adrenalectomized rats.

Adrenalectomized rats maintained with DCA exhibit hyperketonemia and hypoglycemia during a four day fast.

Cortisone treatment restores the ketonemia and glycemia of adrenalectomized rats to normal.

The significance of these findings in relation to the roles of the circulation and metabolism after adrenalectomy and in terms of current concepts of the role of the adrenal cortex in fat and carbohydrate metabolism is discussed.

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# ROLE OF THE THYROID AND ANTERIOR PITUITARY GLANDS IN THE MAINTENANCE OF THE HARDERIAN GLANDS OF THE RAT

NORMAN F. BOAS AND ROBERT W. BATES

*National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland*

THE Harderian gland, a retro-orbital gland, is present in reptiles, amphibians and in many species of mammals, but not in man. It assumes relatively large size in rats (Venable and Grafflin, 1940) and in guinea pigs, where it has received most study. It is a distinctive gland because of its high porphyrin and fat content.

Little is known in regard to endocrine influences on this gland. Smelser (1943) and Pochin (1945) have shown that intact or thyroidectomized guinea pigs treated with anterior pituitary extracts develop hypertrophy of the Harderian glands, suggesting a role of the anterior pituitary in their maintenance. During the course of studies in experimental exophthalmos in rats (Boas and Scow, 1954), it was observed that the Harderian glands atrophied following either thyroidectomy or hypophysectomy. Studies were therefore carried out in an attempt to assess the roles of the thyroid, pituitary and other endocrine glands in controlling the Harderian gland in the rat.

## METHODS

Male Sprague-Dawley rats were individually caged, fed a modification of McCollum's lactation diet (Boas and Peterman, 1953), and permitted to drink water or 0.9% saline (adrenalectomized rats) *ad libitum*. Operations were performed under ether, or combined ether-pentobarbital anesthesia. All hypophysectomized and adrenalectomized rats and their controls received daily subcutaneous injections of 5000 units of penicillin. Completeness of hypophysectomy was determined post mortem by examining the sella turcica under magnification. Only the data from completely hypophysectomized rats are presented.

All hormone preparations were injected subcutaneously in a volume of 0.2 ml. Testosterone (free) and estradiol were prepared in sesame oil; cortisone acetate was used as an aqueous suspension; thyroxin was dissolved in water adjusted to pH 9–10. The adrenocorticotrophin (ACTH),[1] thyrotrophin (TSH),[2] and fraction B-1 of the anterior pitui-

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[1] ACTHAR, Armour Laboratories.

[2] TSH, Lot No. 129. 31PRR3, kindly supplied by Dr. Irby Bunding, Armour Laboratories, Chicago.

tary[3] were all formulated in corn oil with 2% aluminum monostearate. Growth hormone (GH)[4] was dissolved in water. All controls received 0.2 ml. injections of physiological saline.

At the termination of each experiment the rats were etherized and exsanguinated by cutting a carotid artery. The Harderian glands were removed from each eye socket, freed of adherent connective tissue, and the pairs weighed. Their water content was determined by re-weighing the glands after drying for 15 hours in an oven at 102–104° C. Fat content was determined by the difference between the dry weight and the weight after extraction for 3 hours in a Soxhlet apparatus with petroleum ether (B. P. 65–75° C). The defatted glands were referred to as the residue.

The glands used for histological study were fixed in formalin and sections were stained with each of the following: hematoxylin and eosin, orange red 0, mucihematein, mucicarmine and periodic acid Schiff reagent.[5]

## RESULTS

*Normal Growth.*—The relationship between the weight of the Harderian glands and body weight is shown in Figure 1. The curve is based on weights from 121 rats which had been used as control groups in several experiments, each solid dot representing the mean of a group of 4 to 10 rats. It is seen that the growth rate of the Harderian glands is less than that of the body. The Harderian glands-body weight ratio (mg. per 100 gm. body weight), which is 160 in the 50 gram. rat, gradually decreases to 102 in the 300 gram. rat.

*Adrenalectomy and Cortisone Treatment.* Four groups of rats were used to evaluate the relationship between the adrenals and Harderian glands. They consisted of an intact control group, an untreated adrenalectomized group, and two adrenalectomized groups receiving daily subcutaneous injections of 2.5 and 0.1 mg. of cortisone acetate, respectively. Treatment was started on the day of operation and the rats were killed 7 days later. The data are shown in Table 1. Neither adrenalectomy nor cortisone treatment significantly altered the weight or composition of the Harderian glands, despite significant body and thymus weight changes in the treated groups. The group receiving the largest dose of cortisone had a high Harderian glands-body weight ratio. This was primarily due to the marked body weight loss in this group.

*Castration and Testosterone Treatment.* Three groups of rats were used to evaluate the relationship between the testes and Harderian glands. They

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[3] B-1. Crude fraction prepared from fresh beef anterior pituitary glands. Glands were generously supplied by Dr. Irby Bunding, Armour Laboratories, Chicago.

[4] GH. No. $C_5I_5$, E. R. Squibb and Sons. This preparation at a dose level of 0.160 mg./day, induced an increase in body weight of 2.5 gm./day in 100 gm. hypophysectomized rats. It contains 0.3 USP units of TSH/mg. and less than 0.2 I.U. of Prolactin /mg.

[5] We wish to thank Dr. James B. Longley for preparing the histological material.

TABLE 1. EFFECTS OF ADRENALECTOMY AND CASTRATION AND TREATMENT WITH CORTISONE AND TESTOSTERONE ON THE HARDERIAN GLANDS OF THE RAT*

| Group | Total dose (mg.) | No. rats | Body wt. (gm.) | Harderian glands (mg.) | Harderian glands (mg.) / 100 gm. body wt. | Water (%) | Fat (%) | Residue (%) | Thymus (mg.) | Seminal vesicles (mg.) |
|---|---|---|---|---|---|---|---|---|---|---|
| Control | — | 5 | 148±3 | 185±14 | 124±9 | 67.3±1.3 | 15.2±1.0 | 17.5±0.9 | 530±35 | — |
| Adrenalectomy | — | 7 | 143±4 | 183± 5 | 128±4 | 67.5±0.9 | 15.3±1.2 | 17.2±0.4 | 659±72 | — |
| Adrenalectomy +Cortisone | 17.5 | 7 | 105±4 | 161±10 | 154±8 | 65.8±1.1 | 18.3±1.3 | 15.9+0.8 | 56± 5 | — |
| Adrenalectomy +Cortisone | 0.7 | 5 | 135±8 | 180±19 | 133±9 | 70.3±1.0 | 15.1±0.8 | 14.6±0.4 | 573±27 | — |
| Control | — | 5 | 138±4 | 181± 4 | 132±5 | 65.5±1.0 | 18.1±1.3 | 16.4±0.5 | — | 94±16 |
| Castration | — | 5 | 134±6 | 177± 7 | 133±2 | 67.1±1.7 | 17.3±1.6 | 15.6±0.1 | — | 25± 4 |
| Castration +Testosterone | 6.0 | 5 | 128±3 | 173± 5 | 136±3 | 65.4±1.1 | 17.8±1.8 | 16.8±0.8 | — | 45± 5 |

* Duration of each experiment was 7 days. Data presented as Mean ± Standard Error.

consisted of an intact control group, and 2 castrated groups, one untreated and one receiving subcutaneous injections of 3 mg. of testosterone on the day of operation and the 4th post-operation. All rats were killed 7 days following the day of operation. The data in Table 1 indicate that neither

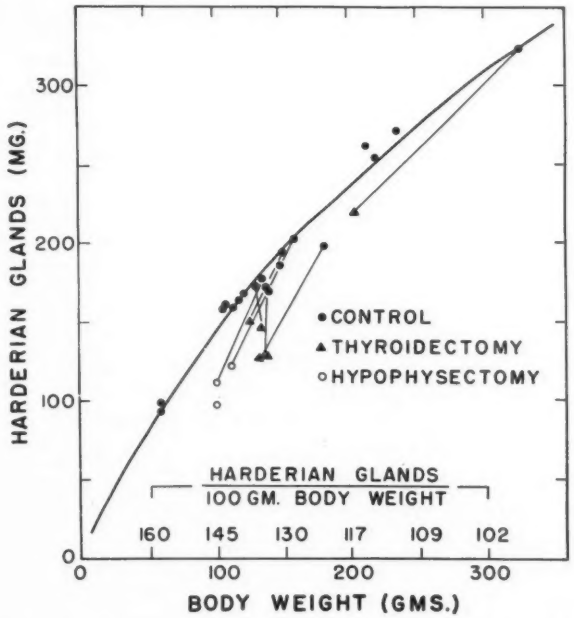


FIG. 1. Relationship between weight of body and Harderian glands in normal, thyroidectomized and hypophysectomized rats. Straight lines connect the operated groups with their unoperated controls of the same age.

TABLE 2. EFFECT OF THYROXIN AND GROWTH HORMONE ON THE HARDERIAN GLANDS OF THYROIDECTOMIZED (Tx) RATS*

| Group | Total dose (mg.) | No. rats | Body wt. (gm.) | Harderian glands (mg.) | Harderian glands (mg.) / 100 gm. body wt. | Water (%) | Fat (%) | Residue (%) |
|---|---|---|---|---|---|---|---|---|
| Intact Control | — | 3 | 148±9 | 192±5 | 130±7 | 67.5±0.8 | 17.1±1.2 | 15.5±0.5 |
| Tx- | — | 4 | 127±10 | 153±5 | 122±9 | 67.1±0.5 | 16.3±0.9 | 16.6±0.5 |
| Tx-Thyroxin | 0.021 | 5 | 137±8 | 183±9 | 136±10 | 67.1±1.2 | 17.7±1.3 | 15.2±0.5 |
| Tx-Thyroxin+GH | 0.021<br>3.5 | 5 | 144±5 | 192±6 | 134±4 | 64.2±0.5 | 19.5±0.8 | 16.3±0.4 |
| Tx-GH | 3.5 | 5 | 135±6 | 160±9 | 120±7 | 68.4±0.6 | 15.9±0.5 | 15.7±0.6 |

* Duration of experiment was 7 days. Data presented as Mean ± Standard Error.

castration nor testosterone treatment effected any significant changes in the weight or composition of the Harderian glands, despite significant changes in seminal vesicle weight.

Estradiol was ineffective in inducing any Harderian gland changes in hypophysectomized rats (Table 3).

*Thyroidectomy.*—To study the influence of the thyroid on the Harderian glands, 31 rats weighing 110–120 gm. were used: five as a zero day control group; the remainder were thyroidectomized (14) or sham thyroidectomized (12). Four or 5 rats in each group were killed at 4, 8, and 16 days, and the Harderian glands weighed. Atrophy of the glands was evident within a few days following thyroidectomy (Fig. 2). Maximal atrophy occurred at 8 days with no further decrease thereafter. The decrease in the Harderian gland-body weight ratio was also greatest at 8 days and was 88±7 mg./100 gm. body weight as compared with 122±14 mg./100 gm. body weight for the 8-day controls.

Since body growth of thyroidectomized animals was considerably retarded relative to controls of the same age, body weight was considered in evaluating changes in Harderian gland weights. The data from 5 different experimental groups of thyroidectomized rats were superimposed on the normal growth curve in Figure 1. A total of 27 thyroidectomized rats is represented, each point being the mean of a group of 4–9 rats. The connecting lines represent the pairing of each group with its control group of the same age, the slopes of the lines depending on the duration of each experiment, which varied from 4 to 72 days.

These data demonstrated that not only was there an absolute weight decrease of the Harderian glands following thyroidectomy, but that this decrease was disproportionate to body weight. This suggested that a substance or substances trophic to the Harderian glands were lost following thyroidectomy.

The composition of the Harderian glands changed following thyroidectomy. The typical changes are shown in Figure 3. These data represent a comparison between two groups of rats killed 8 'ays following thyroidec-

TABLE 3. EFFECTS OF THYROXIN, ESTRADIOL AND HORMONES OF THE ANTERIOR PITUITARY GLAND ON THE HARDERIAN GLANDS OF HYPOPHYSECTOMIZED (Hx) RATS*

| Group | Total dose | No. rats | Body wt. (gm.) | Harderian glands (mg.) | Harderian glands (mg.) / Body wt. 100 gm. | Water (%) | Fat (%) | Residue (%) | Adrenals (mg.) | Testes (mg.) | Thyroid (mg.) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Intact control | — | 6 | 135 ± 6 | 176 ± 9 | 130 ± 5 | 67.7 ± 1.2 | 17.4 ± 1.2 | 14.9 ± 0.4 | 28 ± 1.2 | 1740 ± 100 | 9.5 ± 0.7 |
| Hx- | — | 6 | 99 ± 4 | 110 ± 5 | 111 ± 6 | 72.2 ± 0.6 | 10.9 ± 0.8 | 16.9 ± 0.4 | 15 ± 1.2 | 830 ± 45 | 5.7 ± 0.2 |
| Hx-ACTH | 14 Units† | 7 | 74 ± 4 | 124 ± 7 | 169 ± 5 | 66.8 ± 0.4 | 14.0 ± 1.4 | 19.2 ± 1.0 | 51 ± 2.3 | — | — |
| Hx-B-1 | 14 mg.† | 7 | 106 ± 3 | 147 ± 5 | 139 ± 5 | 65.7 ± 0.6 | 19.1 ± 1.0 | 15.2 ± 0.5 | 16 ± 0.7 | 1540 ± 98 | 6.2 ± 0.2 |
| Hx-Thyroxin | 0.018 mg. | 6 | 95 ± 3 | 113 ± 6 | 120 ± 5 | 69.8 ± 1.0 | 14.8 ± 1.1 | 15.4 ± 0.3 | — | — | — |
| Hx-Estradiol | 0.093 mg. | 5 | 87 ± 2 | 98 ± 6 | 113 ± 6 | 73.3 ± 1.0 | 11.3 ± 1.3 | 15.4 ± 0.4 | — | — | — |
| Hx.- | — | 4 | 100 ± 4 | 98 ± 5 | 98 ± 2 | 70.7 ± 1.0 | 11.0 ± 0.8 | 18.3 ± 0.7 | 14 ± 1.7 | 520 ± 154 | 6.2 ± 0.1 |
| Hx-Thyroxin | 0.168 mg. | 3 | 96 ± 5 | 135 ± 6 | 140 ± 2 | 68.4 ± 1.3 | 13.8 ± 1.5 | 17.8 ± 0.7 | — | — | — |
| Hx-TSH | 21 mg.† | 4 | 104 ± 8 | 150 ± 3 | 147 ± 10 | 64.4 ± 1.7 | 19.0 ± 2.4 | 16.8 ± 0.9 | 22 ± 2.6 | 1445 ± 140 | 11.1 ± 1.0 |
| Hx-GH | 14 mg. | 3 | 135 ± 2 | 163 ± 11 | 120 ± 6 | 67.0 ± 0.4 | 15.3 ± 0.4 | 17.7 ± 0.1 | 18 ± 0.8 | 1207 ± 23 | 10.9 ± 1.2 |

* Duration of each experiment was 7 days. Data presented as Mean ± Standard Error.
† In corn oil with 2% aluminum monostearate.

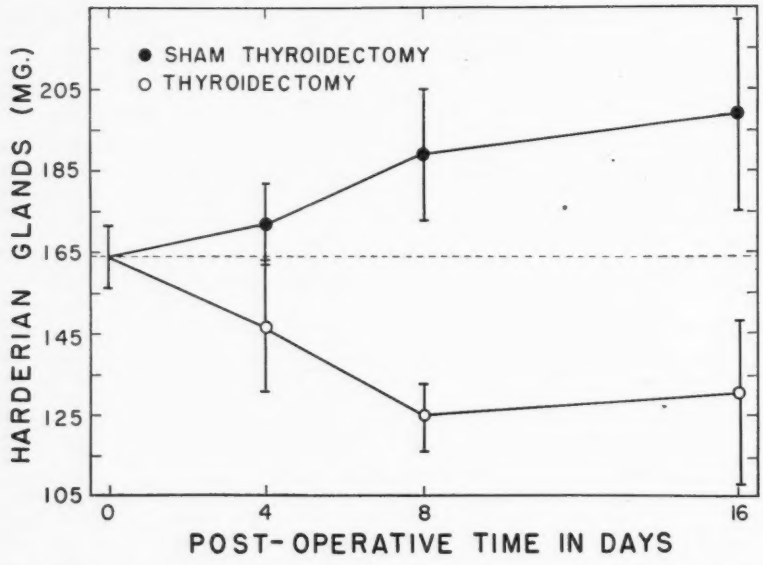


FIG. 2. Effect of thyroidectomy on weight of Harderian glands. Vertical lines refer to S.E. of the Mean.

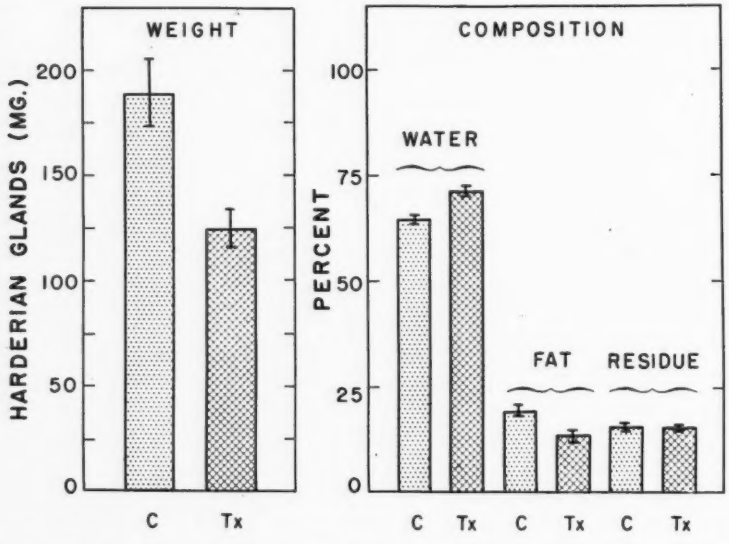


FIG. 3. Effect of thyroidectomy (Tx) on weight and composition of Harderian glands. C refers to sham thyroidectomized controls. The vertical lines refer to S.E. of the Mean.

tomy (5 rats) and sham thyroidectomy (4 rats). The glands from the thyroidectomized group were smaller and on analysis had higher water and lower fat concentrations than the controls. The proportion of residue remained unchanged. These changes in composition have been observed in all thyroidectomized animals studied as early as 4 days and as late as 48 days following thyroidectomy.

*Thyroidectomy.—Treatment with Thyroxin and Pituitary Hormones.* Since the weight of the Harderian glands could not be maintained following thyroidectomy, the deficiency of two possible trophic factors were considered, thyroid hormone and an anterior pituitary factor. It was reasoned that the normal production of certain pituitary hormones may depend on circulating thyroid hormone.

Five groups of animals were used, an intact control group, an untreated thyroidectomized group, and groups of thyroidectomized rats getting thyroxin, growth hormone and thyroxin plus growth hormone. All received subcutaneous injections daily for 7 days from the day of operation. The data are shown in Table 2. The Harderian glands were adequately maintained on only 3 $\mu$g. of thyroxin daily. Growth hormone had no effect alone or in combination with thyroxin. The endogenous production of pituitary hormones may be sufficient to obscure any effect of exogenous G.H. The fat and water changes were not as marked as in previous or subsequent experiments.

*Hypophysectomy.*—Since Harderian gland atrophy resulted from thyroidectomy, hypophysectomized rats were also studied. Rats weighing 120–140 gm. were used. The unoperated controls (8 rats) and hypophysectomized rats (8 rats) were killed 7 days following the day of hypophysectomy (Fig. 4). The hypophysectomized rats demonstrated a significant decrease in the weight of the Harderian glands. As in the thyroidectomized rats, the Harderian glands contained more water and less fat than the controls. The Harderian glands-body weight ratio, $129 \pm 3$ mg./100 gm. in the control group was $109 \pm 2$ mg./100 gm. in the hypophysectomized group. With their eyelids removed the hypophysectomized animals appeared enophthalmic.

The data from 3 different experimental groups of hypophysectomized rats were superimposed on the normal growth curve (Fig. 1). A total of 18 rats is represented, each point being the mean of a group of 4–8 rats. Two of the groups had controls of the same age to which they were paired with straight lines. It was apparent that the Harderian glands were disproportionately small relative to body weight, suggesting that a substance or substances trophic to the Harderian glands were lost following hypophysectomy.

Histological studies were made of the Harderian glands of representative

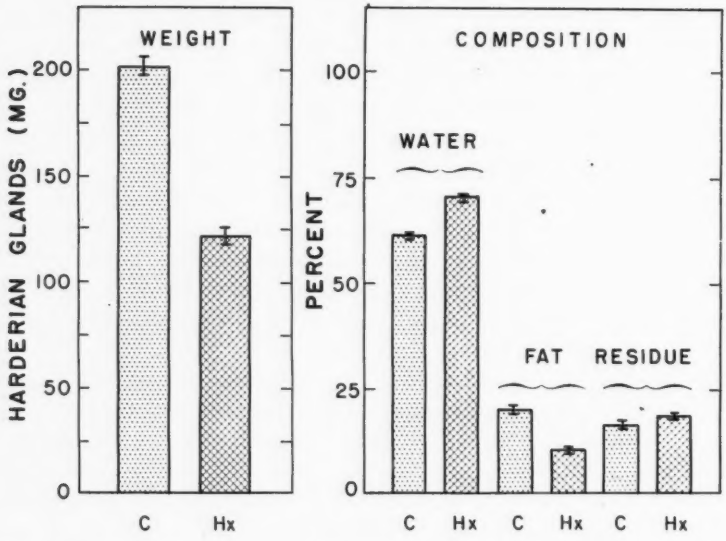


FIG. 4. Effect of hypophysectomy (Hx) on weight and composition of Harderian glands. C refers to the control group of the same age. The vertical lines refer to S.E. of the Mean.

control and hypophysectomized rats. Detailed descriptions of the normal histology have been made by Duthie (1933–4) and Grafflin (1942) and therefore will not be repeated here. The normal glands demonstrated large amounts of intracellular fat and intraluminal porphyrin bodies. No periodic acid Schiff or mucous staining intracellular structures could be seen. The glands from the hypophysectomized animals in general had smaller cells and larger lumina, and appeared to contain decreased amounts of stainable intracellular fat. No other specific abnormalities were noted.

*Hypophysectomy.—Treatment with Thyroxin and Pituitary Hormones.* Atrophy of the Harderian glands resulted from either thyroidectomy or hypophysectomy. Although thyroxin was able to maintain the Harderian

TABLE 4. EFFECT OF STRESS (FRACTURE) ON HARDERIAN GLANDS OF RAT*

| Days after fracture | No. rats | Body wt. (gm.) | Harderian glands (mg.) | Harderian glands (mg.) / 100 gm. body wt. | Water (%) | Fat (%) | Residue (%) |
|---|---|---|---|---|---|---|---|
| 0 | 7 | 105±3 | 157±10 | 149±7 | 68.5±1.2 | 16.4±1.2 | 15.1±0.2 |
| 1 | 7 | 113±4 | 159±7 | 142±8 | 65.3±1.3 | 19.5±1.1 | 15.2±0.4 |
| 2 | 7 | 107±3 | 158±9 | 148±10 | 68.3±1.3 | 17.1±1.0 | 14.6±0.5 |
| 4 | 7 | 120±4 | 166±5 | 139±4 | 64.5±1.1 | 20.2±0.7 | 15.3±0.5 |
| 9 | 6 | 137±5 | 171±10 | 125±10 | 66.6±0.8 | 18.0±0.8 | 15.4±0.3 |

* Mean ± Standard Error.

glands in thyroidectomized rats and exogenous growth hormone had no effect, the possibility still remained that thyroxin stimulated the endogenous production of a pituitary factor which was trophic to the Harderian glands. In order to assess the role of the anterior pituitary, several pituitary preparations and thyroxin were administered to hypophysectomized rats.

Commencing on the day of operation, groups of hypophysectomized rats were treated for 7 days with ACTH, thyroxin, estradiol, or a crude beef anterior pituitary preparation (B-1) which was known to contain gonadotrophic, thyrotrophic, and somatotrophic activity. The data are given in the upper half of Table 3. The effect of hypophysectomy is evident by comparing the hypophysectomized group with the intact control group. Again, as in Figure 4, the glands in the hypophysectomized group were small with relatively more water and less fat. Thyroxin at a dosage level of 2.5 $\mu$g. daily did not significantly influence the weight or composition of the Harderian glands, whereas, the B-1 fraction significantly increased the Harderian gland weight, the Harderian glands-body weight ratio, lowered the water content and increased the fat content to the normal range. ACTH did not increase Harderian gland weight but did cause a striking increase in the Harderian gland-body weight ratio, similar to the effect of cortisone (Table 1) and due primarily to a marked loss in body weight. This increased ratio indicates that the Harderian glands were spared from the catabolic effect of ACTH. The water content was reduced by ACTH, but the fat content was not significantly altered. These ACTH-treated rats had very large adrenal glands averaging 51 mg.

Because of the negative results with estradiol, testosterone, cortisone and ACTH and a positive response with the B-1 fraction known to contain thyrotropin and growth hormone, it seemed possible that one of these two hormones might be the active principle trophic to the Harderian gland. Because of the negative results with thyroxin (2.5 $\mu$g./day), a larger dose was tried.

In the next experiment, four groups of hypophysectomized rats were used, a control group and groups injected with thyroxin, TSH, and GH. The animals received daily subcutaneous injections for 7 days starting on the day of the operation. The data are shown in the lower half of Table 3. All treated groups had significantly larger Harderian glands with higher gland-body weight ratios and shifts in water and fat contents approaching normal. Despite the large (toxic) dose of thyroxin given (24 $\mu$g./day) the changes in the Harderian glands were not as striking as with the TSH and GH preparations. Both the TSH and GH preparations gave evidence of gonadotrophic, ACTH and TSH activity.

*Stress.* Since maintenance of the Harderian glands appeared to be under pituitary-thyroid control, a study of the response of the Harderian glands

to stress was made as a possible measure of pituitary-thyroid activity following stress. The right femur and tibia were fractured in 26 rats under light ether anesthesia. An initial control group (7 rats) was used. The fractured animals were sacrificed in groups at 1, 2, 4 and 9 days and the Harderian glands removed. No significant changes resulted in the weight or composition of the Harderian glands.

## DISCUSSION

The Harderian glands of thyroidectomized or hypophysectomized rats were consistently smaller than those of controls of the same age. This was shown to result from an initial post-operative atrophy and subsequent failure to grow. The weight of the Harderian glands was unchanged by removal of the testes, adrenals or therapy with estradiol, testosterone, cortisone or ACTH.

The atrophy and growth failure of the Harderian glands following hypophysectomy could be prevented by the administration of either a TSH preparation (contained GH) or a GH preparation (contained TSH), and partially prevented by a large dose of thyroxin. A small maintenance dose of 2.5 $\mu$g/day of thyroxin was ineffective. On the other hand, the atrophy and growth failure resulting from thyroidectomy could be prevented with a similar small dose of thyroxin but not with pituitary extract alone.

The presence of an intact pituitary therefore seemed necessary for thyroxin to act and the presence of an intact thyroid was necessary for the pituitary fractions to work. Maintenance of the Harderian glands therefore appeared to depend on one of two mechanisms, 1) a synergism between the action of thyroxin and a pituitary factor, possibly growth hormone, or 2) a pituitary factor, the release of which was dependent upon the presence of thyroxin.

The exact nature of the pituitary factor has not been established. It is not ACTH or a gonadotrophin, and is not simply a thyrotrophic factor since maintenance doses of thyroxin would not prevent Harderian gland atrophy in the hypophysectomized rat. Two pituitary fractions containing growth hormone were effective in maintaining Harderian gland weight in hypophysectomized rats, but this does not preclude the possibility of other unidentified pituitary factors, playing a role.

Smelser (1938, 1943) has shown that (1) intact guinea pigs develop hypertrophy of the Harderian glands following stimulation with crude anterior pituitary extracts, (2) thyroidectomized guinea pigs develop hypertrophy without hormone treatment, (3) with pituitary extracts thyroidectomized guinea pigs develop Harderian gland hypertrophy greater than seen in similarly treated intact controls, and (4) that treatment of intact or thyroidectomized guinea pigs with thyroid hormone produces a reduction

in Harderian gland size. These data suggest that thyroid hormone acts only as an inhibitor to a pituitary factor trophic to the Harderian glands. These guinea pig Harderian glands developed a true hypertrophy, whereas Harderian glands of rats under the conditions presented here, when stimulated, never exceeded a normal weight. Although the rats were treated for only 7–8 days, and Smelser's guinea pigs were treated for at least 30, and sometimes as long as 60 days, the difference in the duration of the experiments would not necessarily explain the failure of hypertrophy in the rat. It has been shown that hypertrophy of the guinea pig Harderian glands can be induced within 7 days (Pochin, 1945). Stimulation of guinea pig glands by pituitary extracts produced a decrease in fat and an increase in water content, whereas in the rat the reverse was true.

It is difficult to reconcile Smelser's observations on the Harderian glands of the guinea pig with those reported here in the rat. In both species the Harderian glands can be stimulated by an anterior pituitary factor. The rat also requires thyroid hormone, whereas Smelser's data indicate that thyroid hormone in the guinea pig is not essential, since thyroidectomy actually augments the response of the Harderian glands to pituitary extracts. It is likely that the observed differences between the guinea pig and rat represent a species difference.

Neither pregnant mare serum nor estradiol increased Harderian gland weight in the guinea pig (Smelser, 1943). Estradiol has actually been reported to cause atrophy of the Harderian gland (Smelser, 1943; Donatelli and Benelli, 1946).

## SUMMARY

The Harderian gland-body weight ratio in the rat decreases with age. Weight of the Harderian gland is not changed by adrenalectomy, castration, or stress (fracture), nor is it changed by treatment with ACTH, cortisone, estradiol or testosterone.

Following either thyroidectomy or hypophysectomy, the Harderian glands of the growing rat atrophy and do not grow appreciably thereafter.

The atrophy and growth failure following hypophysectomy can be prevented by the administration of pituitary fractions containing growth hormone and TSH, partially prevented with a large dose of thyroxin but not with a maintenance dose of thyroxin.

The atrophy and growth failure following thyroidectomy can be prevented by maintenance doses of thyroxin, but not with mixtures of anterior pituitary hormones.

It is suggested that normal maintenance of the Harderian glands in the rat depends upon the presence of thyroid hormone and an anterior pituitary factor trophic to the Harderian glands, possibly growth hormone.

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# ANALYSIS OF THE ACTION OF PROPYLTHIOURACIL ON THE PITUITARY-THYROID AXIS OF RATS[1]

N. S. HALMI AND B. N. SPIRTOS

*Department of Anatomy, State University of Iowa, Iowa City, Iowa*

THERE is no doubt that the primary action of propylthiouracil (PTU) and other drugs of the thiocarbamide series on the thyroid is an interference with the iodination of tyrosine. It is also well established that the ensuing hypothyroid condition evokes compensatory hypersecretion of thyrotrophin (TSH) resulting in two characteristic effects of enhanced thyrotrophic stimulation of the thyroid: a) hypertrophy and hyperplasia of the thyroid epithelium (Astwood, 1944–45), and b) activation of the thyroidal iodide concentrating mechanism, as shown by an increase in the thyroid: serum iodide gradient (VanderLaan and VanderLaan, 1947; Taurog *et al.*, 1947) and an expanded capacity of the gland for iodide (Halmi, 1954a). In general goitrogenesis and activation of the iodide concentrating mechanism induced by antithyroid agents go hand in hand, but rats bearing hypothalamic lesions and subjected to PTU treatment may display a rise of the iodide gradient without a concomitant goitrous response of the thyroid (Greer, 1951, 1952; Bogdanove and Halmi, 1953). Greer (1952) sought to explain this dichotomy by hypothesizing that two thyrotrophic principles are elaborated by the pituitary, one promoting thyroid growth and the other thyroidal iodine uptake. Halmi *et al.* (1953), on the other hand, suggested that PTU may selectively potentiate the effect of TSH on the iodide concentrating mechanism, since they found that when TSH was given to hypophysectomized rats, the activation of the iodide concentrating mechanism, but not the morphological response of the thyroid, was greatly enhanced if PTU was also administered. These findings were confirmed by VanderLaan and Caplan (1954).

Rawson and Money (1949) had previously proposed that goitrogens effect an over-all augmentation of TSH activity. This concept was based on observations of Albert *et al.* (1947), according to which thiouracil, in doses not sufficiently high to cause thyroid enlargement, enhanced the response (as measured by weight changes and iodine loss) of the chick thyroid to TSH. The study of Albert *et al.* is open to criticism, as pointed out by the authors themselves, since the experimental animals were not hypophysec-

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tomized. However, unpublished work by Cortell (quoted by Rawson and Money, 1949) and a somewhat poorly documented paper by Wessels *et al.* (1950) seem to indicate that even under more rigorous experimental conditions, i.e. if hypophysectomized rats are used as test animals, greater thyroid cell height increments can be achieved by giving TSH plus goitrogens than with TSH alone.

Since the response of the iodide concentrating mechanism to TSH is more sensitive than is that of follicular morphology (VanderLaan and Greer, 1950; Halmi, 1954a; VanderLaan and Caplan, 1954), it could be argued that the apparently selective enhancement by PTU of the action of TSH on the iodide concentrating mechanism was observed by Halmi *et al.* (1953) and by VanderLaan and Caplan (1954), was merely the manifestation of an over-all potentiation of TSH given in doses too low to affect thyroid cell height. In order to rule out this possibility, follicular cell heights and thyroid:serum iodide concentration ratios were simultaneously determined in hypophysectomized rats receiving graded doses of TSH with or without concomitant PTU treatment. Furthermore, PTU was administered to hypophysectomized rats not given TSH to ascertain whether the goitrogen *per se* has any effect on the iodide concentrating mechanism.

## MATERIAL AND METHODS

All rats used in this study were hypophysectomized males (150–180 gm.) of the Sprague-Dawley strain, obtained 8 days postoperatively from the Hormone Assay Laboratories, Chicago. In *Experiment I* the rats were fed Purina Laboratory Chow supplemented with fresh oranges and 20 days after the operation were divided into four groups which received respectively: a) no treatment, b) propylthiouracil (PTU) in suspension form (10 mg. daily for 5 days) injected subcutaneously, c) the same dosage of PTU together with TSH (.05–1.0 U.S.P. units of U.S.P. Thyrotropin Reference Substance per day given subcutaneously for 5 days),[2] and d) TSH (.05–2.0 U.S.P. units daily for 5 days) alone. All rats in this experiment were sacrificed 26 days postoperatively. Thyroid-serum radioiodide concentration (T/S) ratio determinations and follicular cell height measurements were carried out as described in earlier studies (Halmi *et al.*, 1953; Halmi, 1954b). In *Experiment II* the rats were divided into two groups and maintained on Purina Laboratory Chow. One group of animals was injected with 10 mg. of PTU (as suspension) daily for 10 days; the control group was not treated. T/S ratio determinations were performed 19 days postoperatively.[3]

## RESULTS

The results of *Experiment I* are summarized in Table 1 and Figure 1.

Propylthiouracil (PTU), when given to hypophysectomized rats in the dosage of 10 mg. daily for 5 days, failed to cause a rise in thyroid follicular cell height. All doses of thyrotrophin (TSH) tested effected a significant in-

[2] Care was taken to inject the two substances in different sites.

[3] Thyroid lobes saved for histological examination were accidentally lost.

TABLE 1. EFFECT OF GRADED DOSES OF THYROTROPHIN (TSH) WITH OR WITHOUT PROPYLTHIOURACIL (PTU) ON THYROID:SERUM IODIDE CONCENTRATION (T/S) RATIOS AND MEAN THYROID CELL HEIGHTS IN HYPOPHYSECTOMIZED RAT

| Dose TSH* | Number of rats | | T/S ratio | | Mean thyroid cell height, μ | |
|---|---|---|---|---|---|---|
| | No PTU | PTU | No PTU | PTU | No PTU | PTU |
| 0 | 9 | 10 | 4.5± .57† | 6.5± .43 | 3.7±.10 | 3.4±.09 |
| .05 | 12 | 11 | 73.4± 8.6 | 194.6±15.9 | 4.6±.09 | 5.0±.22 |
| .3 | 12 | 8 | 111.4± 6.5 | 278.4±25.0 | 5.3±.14 | 6.3±.17 |
| 1.0 | 8 | 8 | 94.0± 9.0 | 417.5±12.2 | 7.9±.21 | 8.8±.18 |
| 2.0 | 10 | — | 139.2±15.8 | — | 9.0±.24 | — |

* U.S.P. units per day for 5 days.
† Mean ±standard error.

crease in acinar cell height. In the range of .3-2.0 U.S.P. units daily for 5 days the log dose-response curve was rectilinear. The cell height increments after the simultaneous administration of PTU and TSH were, at the three dose levels examined, greater than those elicited by TSH alone. The difference was statistically highly significant ($P < .01$) at the dose levels of .3

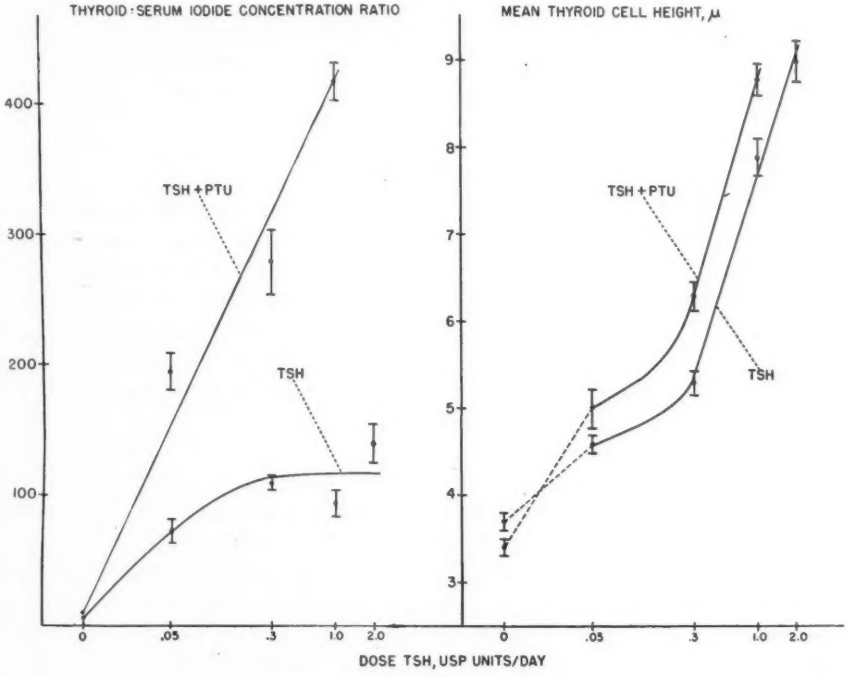


FIG. 1. Semilogarithmic plots of the data summarized in Table 1. The vertical lines indicate standard errors.

and 1.0 U.S.P. unit per day. The "log dose TSH-cell height response" curve for TSH plus PTU paralleled that obtained for TSH alone, which suggests that the goitrogen had a quantitative influence in augmenting the action of the hormone. Since the mean follicular cell height in the rats treated with 2.0 U.S.P. units of TSH daily was not significantly different from that of animals receiving 1.0 U.S.P. unit daily together with PTU, it can be concluded that the effectiveness of TSH, as judged by morphological criteria, was approximately doubled by concomitant PTU treatment of the recipients.

On the other hand, the "log dose TSH-thyroid serum iodide gradient response" curves for the TSH treated animals and those receiving TSH plus the goitrogen were altogether dissimilar. Whereas the thyroid:serum iodide concentration (T/S) ratio of the rats treated with the hormone alone apparently reached a plateau at the .3 U.S.P. unit dose level and failed to rise significantly above 120 even when the dosage was further increased, the gradient in the rats given TSH plus PTU did not level off within the tested dose range. At the 1.0 USP unit level the T/S ratio was over 400 and thus exceeded by more than three times the gradient in the animals receiving 2.0 U.S.P. units of TSH alone, although the thyroid cell height of these two groups of animals was similar (8.8 vs. 9.0 $\mu$). Furthermore, in the animals given even the lowest dosage of TSH in conjunction with the goitrogen, the T/S ratio was significantly greater than in those receiving the highest dose of TSH alone, albeit the follicular cell height of the latter group was almost double that of the former (9.0 vs. 5.0 $\mu$).

The T/S ratio of the rats given PTU alone for 5 days was significantly higher than that of untreated controls; it was nevertheless comparable with values previously found in hypophysectomized rats which had not received prolonged goitrogen treatment (Halmi *et al.*, 1953). In *Experiment II*, however, the injection of 10 mg. of PTU daily for 10 days brought about a rise in the T/S ratio to values unequivocably higher than the range for untreated hypophysectomized animals ($16.0 \pm 3.6$[4] in 16 treated rats as compared with $5.0 \pm .63$[4] in 28 uninjected controls).

## DISCUSSION

The morphological observations reported in this paper lend firm support to the thesis of Rawson and Money (1949) that goitrogens not only increase the output of thyrotrophin (TSH) by the pituitary but also augment the effectiveness of the hormone. We believe that the evidence offered by our observations is cogent because a) hypophysectomized rats were used, whereby endogenous hormone effects were eliminated, and b) TSH,

[4] Mean ± standard error.

with or without propylthiouracil (PTU), was given in graded doses; it could therefore be demonstrated that PTU enhanced the cell height response to all doses of the hormone tested and that the slope of the dose-response curve was similar whether or not PTU was given. These morphological findings are compatible with the idea that goitrogen treatment has a simple quantitative over-all potentiating effect on the activity of TSH. It appears most likely that this is due to a diminished rate of destruction and/or elimination of TSH. D'Angelo (1953) has shown that the biological half life of injected TSH is greater in goitrogen treated rats than in controls. Although Rawson and Money (1949) have strongly advocated a primary role of the thyroid in the inactivation of TSH, it cannot be stated with certainty that other tissues do not play an equally or more important part in the destruction of TSH *in vivo*. The sluggish removal of TSH from the blood stream of goitrogen treated animals may be mainly due to the absence of a thyroid-mediated rise in the rate of oxidative metabolism of various tissues under such conditions.

The results reported in this paper also make it evident that an over-all potentiation of TSH by PTU fails to account for the drastic enhancing influence of the goitrogen on the response of the thyroidal iodide concentrating mechanism to TSH. In view of the lack of parallelism between the respective "log dose thyrotrophin—iodide concentrating mechanism response" curves obtained for the animals receiving the combined treatment and for those receiving TSH alone, it appears correct to state that PTU has a qualitatively modifying as well as an augmenting effect on the action of TSH on the iodide concentrating mechanism. However, we do not believe that the goitrogen actually achieves this either by acting on the TSH molecule or by directly priming the iodide concentrating mechanism to the hormone.[5] The apparent direct effect of PTU on the iodide concentrating mechanism also requires explanation.

[5] Although the hypothesis has been advanced that even the influence of TSH on thyroidal iodine uptake is secondary to the effect of the hormone in promoting thyroid hormone discharge (Rawson, 1949), there appears to be circumstantial evidence that TSH does act directly on the iodide concentrating mechanism and not merely through the changes that it elicits in the chemical composition of the thyroid. Hypophysectomy in chronically PTU-treated rats leads to a fall in the thyroid:serum iodide concentration (T/S) ratio even though the administration of the goitrogen is continued (VanderLaan and Greer, 1950). All the iodine in the thyroid of rats on a chronic goitrogen-regimen exists as iodide (VanderLaan and VanderLaan, 1947), and it is most unlikely that other iodine containing compounds could accumulate in the gland after hypophysectomy while the antithyroid drug is being administered. Thus, it appears necessary to attribute the fall in the T/S ratio which appears under these circumstances to the lack of TSH *per se*, and to ascribe to this hormone a direct action on the iodide concentrating mechanism.

In attempting to interpret those influences of PTU on the iodide concentrating mechanism which cannot be due to increased release and over-all augmentation of TSH, one must keep in mind the fact that other agents affecting thyroidal iodine metabolism have similar effects. For instance, low iodine intake exerts some stimulation on the iodide concentrating mechanism in the absence of TSH (Halmi, 1954b), as does PTU. Furthermore, VanderLaan and Caplan (1954) have pointed out that in hypophysectomized rats given TSH, factors other than PTU, such as a low iodine diet or potassium thiocyanate administration and withdrawal, also enhance the reaction of the iodide concentrating mechanism, whereas high iodine intake diminishes it. They made the generalization that in the hypophysectomized animal receiving a standard dose of TSH, the response of the iodide concentrating mechanism is inversely proportional to the total thyroidal iodine concentration. These facts support the concept that the iodide concentrating mechanism is controlled by a "thyroid activating mechanism in addition to thyrotropin" which is "intimately associated with the total iodine concentration of the gland" (Astwood, 1953). The complex chemical nature of total thyroidal iodine should preclude any suggestion that its concentration *per se* is a factor in the regulation of the iodine concentrating mechanism. Moreover, though the level of iodine intake influences the iodide concentrating mechanism of hypophysectomized rats, Goldberg *et al.* (1953) have found no difference between the total iodine concentrations in the thyroids of hypophysectomized rats, whether they were fed an iodine deficient diet or the same regimen supplemented with KI. However, the possibility remains that one of the several organic iodine-containing compounds in the thyroid, whose concentration under most circumstances is assumed to parallel that of total thyroidal iodine, and which could be thyroid hormone proper, is a direct inhibitor of the iodide concentrating mechanism.

The following hypothesis is thus offered to explain the effect of PTU on the iodide concentrating mechanism in the absence of TSH, as well as its augmenting and modifying influence on the response of the iodide concentrating mechanism to TSH:

a) In the hypophysectomized animal there exists a steady state of thyroidal iodine, since both the discharge (Wolff, 1951; Randall *et al.*, 1951) and the uptake (Randall and Albert 1951) of iodine by the gland are diminished. If an agent such as PTU interferes with the collection of iodine without affecting its biological decay, there will be an eventual decrease in the thyroidal concentration of the hypothetical iodide concentrating mechanism-inhibitor, and the thyroid:serum iodide gradient will rise.

b) If TSH is given to a hypophysectomized animal which is under the

influence of an agent interfering with the accumulation of thyroidal iodine, the well-known effect of the hormone in enhancing iodine discharge from the thyroid will rapidly deplete the gland of organic iodine-containing compounds, including the hypothetical iodide concentrating mechanism-inhibitor. There is little change in the concentration of total thyroidal iodine (and presumably that of the iodide concentration mechanism-inhibitor) when TSH alone is administered to hypophysectomized rats (VanderLaan and Greer, 1950). The iodide concentrating mechanism is more active in the rats treated with TSH and PTU than in those receiving the hormone alone because in the former group there is lack of inhibition by the intrinsic thyroidal factor as well as direct stimulation of the mechanism by TSH.

In brief, the level of activity of the iodide concentrating mechanism appears to be determined by two factors antagonistic to each other: TSH, the stimulant, and an intrinsic thyroidal principle, the depressant. TSH is envisaged as being capable of enhancing the effectiveness of the iodide concentrating mechanism both by direct action and when thyroidal iodine fixation is handicapped, by diminishing the thyroidal concentration of the depressant.

## SUMMARY AND CONCLUSIONS

Propylthiouracil (PTU) augmented the thyroid cell height response in hypophysectomized rats receiving graded doses of thyrotrophin (TSH), without changing the slope of the log dose TSH-response curve. It enhanced the effectiveness of the hormone on the thyroid:serum iodide concentration (T/S) ratios of the same animals, altering the character of the curve obtained by plotting the response against the log dose of TSH. The goitrogen also elevated the T/S ratio of hypophysectomized rats not receiving TSH.

It is concluded that the hyperplastic changes of the thyroid which are evoked by goitrogen treatment are due to at least two factors: a) increased release of TSH and b) augmentation of its action. In the causation of the concomitant rise of the T/S ratio two further factors seen to be involved: a) a direct action of the drug on the thyroid and b) an apparent selective potentiation of the effect of TSH on the thyroidal iodide concentrating mechanism. It is suggested that PTU exerts the latter two influences by decreasing the thyroidal concentration of a hypothetical inhibitor of the iodide concentrating mechanism, possibly thyroid hormone itself.

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# BLOOD AND PITUITARY ACTH IN INTACT AND ADRENALECTOMIZED RATS AFTER STRESS[1]

KATHERINE L. SYDNOR[2] AND GEORGE SAYERS
With the technical assistance of LURRINE BURGESS AND LAWRENCE HEISELT

*Department of Pharmacology, University of Utah College of Medicine, Salt Lake City, Utah, and Department of Physiology, Western Reserve University School of Medicine, Cleveland, Ohio*

THE time sequence and the mechanism(s) involved in the discharge of ACTH from the adenohypophysis are problems of basic importance to our knowledge of the role of the adrenal cortex in the reaction of the organism to stress. Estimates of ACTH discharge have been based on alterations in the adrenal cortex of the stressed animal at one or more hours after application of the stressful stimulus. Such estimates are of limited value since they are based on the degree of change in the adrenal cortex long after the application of the stimulus. Furthermore, it is entirely possible that stress acting in combination with ACTH will produce an alteration in the concentrations of adrenal metabolites of greater magnitude than that produced by ACTH alone. The development of a technic for the analysis of blood ACTH has overcome these difficulties. This index more nearly reflects pituitary activity at any instant. Samples of blood for analysis may be removed from animals within seconds after application of the stressful stimulus. These samples are processed to concentrate the ACTH activity which is assayed in hypophysectomized animals.

## METHODS

Male rats from the Sprague-Dawley or Holtzman-Rolfsmeyer farm were acclimatized for two weeks in our laboratory under conditions of constant lighting and temperature ($78 \pm 2°$ F.). The animals weighed 200 to 300 gm. at the beginning of the experiments. Adrenalectomized animals were given 0.9% NaCl solution to drink.

Analyses for ACTH were conducted on blood and pituitary obtained from non-

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[2] Present address: Department of Physiology, Western Reserve University School of Medicine, Cleveland, Ohio.

stressed rats and from rats after the application of the following stressful stimuli: (1) exposure to ether, (2) exposure to ether followed by a standardized scald, (3) continuous ether anesthesia plus rapid exsanguination from the abdominal aorta, and (4) continuous ether anesthesia plus repeated bleeding at four-minute intervals. A detailed description of each type of stress is presented below.

A sample of heparinized blood obtained from each rat was added to 4 volumes of glacial acetic acid immediately after collection; the blood-glacial acetic acid mixtures from the rats of each experimental group were pooled and processed by the oxycellulose technic (Sydnor and Sayers, 1951). The HCl-blood eluates were adjusted to pH 4 immediately before assay in hypophysectomized rats by the adrenal ascorbic acid-depletion method (Sayers, Sayers and Woodbury, 1948). Single or multiple doses of unknown were assayed on the same day against multiple doses of U. S. P. ACTH Standard. Concentration is expressed as milliunits[3] (mu.)/100 ml. of blood.

The adenohypophysis from each animal was macerated in 0.5 ml. 0.1 N HCl. Glands from the rats of each experimental group were pooled and stored at 0° C. One day prior to assay the desired concentrations of pituitary were prepared by dilution with 0.01 N HCl. ACTH content of the pooled pituitaries was determined by the adrenal ascorbic acid-depletion method in hypophysectomized rats. Test solutions and U.S.P. ACTH Standard were assayed on the same day. Content is expressed as mu. per pituitary.

*Non-stressed rats.* Each animal was quickly decapitated. Blood from the trunk was collected in a beaker containing a known volume of glacial acetic acid. Decapitation precludes pituitary discharge from contributing ACTH to blood collected from the trunk of the animal. It is assumed that the concentration of ACTH in blood obtained under these experimental conditions represents the concentration of ACTH in the circulation of non-stressed animals.

*Stressed rats.* (1) *Ether anesthesia.* One group of intact and one group of adrenalectomized rats inhaled ether for 45 seconds. Two minutes after initial exposure to ether the animals were decapitated.

(2) *Ether plus scald.* Rats were exposed to ether for 45 seconds; 8 seconds later trunk and extremities were submerged in an aqueous solution of Duponal[4] at $70 \pm 2°$ C. for 7 seconds. They were decapitated one minute, 5 minutes, or $24 \pm 4$ hours after scald.

(3) *Ether plus rapid exsanguination.* Three groups of intact, three groups of rats adrenalectomized for 1 week, and two groups of rats adrenalectomized for 2 weeks were studied. Under continuous ether anesthesia each animal was rapidly exsanguinated via the abdominal aorta into a heparinized syringe. In this experiment, in contrast to the above, the adenohypophysis was intact during bleeding.

(4) *Ether plus repeated bleeding.* Forty-two intact rats were exposed to ether for 11 minutes; 2 to 3-1/2 ml. blood were collected via the aorta into a heparinized syringe during the fourth, the seventh, and the eleventh minute of anesthesia. Each sample was added to 4 volumes glacial acetic acid immediately after collection. The blood-glacial acetic acid mixtures for each collection period were pooled before processing for ACTH. Another experiment was conducted on rats 2 weeks after adrenalectomy. In this experiment 2 to 3 ml. blood were collected during the third, seventh, eleventh and fifteenth minute of anesthesia. In some of these animals blood volume was restored by intra-arterial injection of 2 to 3 ml. 0.9% sodium chloride solution. (Adrenalectomized rats do not tolerate the infusion of hypophysectomized rat blood.)

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[3] One milliunit is equal to one-thousandth of a U.S.P. ACTH (La-1-A).

[4] Duponal (Du Pont de Nemours) is a detergent employed to insure thorough wetting of hair.

## RESULTS

The results of the analyses of blood and pituitary ACTH are presented in Tables 1 and 2, respectively. The data of the individual assays are presented in Appendix I for blood from intact animals, in Appendix II for blood from adrenalectomized animals, and in Appendix III for pituitary.

*Blood ACTH in non-stressed rats.* The titer of ACTH in the blood of non-stressed intact rats is too low to be detected by the technic employed for the concentration and assay of ACTH. A dose of 0.25 mu. ACTH Standard induced a significant depletion of adrenal ascorbic acid. Doses of eluate equivalent to 42 ml. blood from intact rats did not induce a significant depletion of adrenal ascorbic acid (Table 1 and Appendix I, assays 1-a, 2, 3-a and 4-a). It is concluded that the concentration of ACTH in blood of non-stressed intact rats is less than 0.5 mu./100 ml.

In contrast, blood of the non-stressed adrenalectomized rat contains a detectable quantity of ACTH. Estimates based on the analysis of blood obtained from 3 separate groups of rats sacrificed one week after adrenalectomy were $1.6 \pm 0.7$, $3.5 \pm 1.0$ and $3.3 \pm 0.5$ mu./100 ml. (Table 1 and Appendix II, assays 5-a 6-a and 7-a). The weighted mean $\pm$ S.E.M. for the 3 analyses is $3.2 \pm 0.5$ mu.

*Pituitary ACTH in non-stressed rats.* The results of two additional, but separate, experiments (assays 21 and 22) indicate that the pituitary of the non-stressed intact rat contains approximately 70 mu. ACTH. The weighted mean $\pm$ S.E.M. for the two experiments is $75.1 \pm 8.6$ mu./pituitary. These values are in agreement with the estimates based on single-dose assays of pituitaries from animals whose blood was analyzed for ACTH (Table 2 and Appendix III, assays 1-b, 3-c and 4-c). The ACTH content of the pituitary of the rat one week post-adrenalectomy was not significantly different from that of the intact animal (Table 2 and Appendix III, assay 5-e). Two weeks after adrenalectomy the content has increased to 180 mu. The weighted M. $\pm$ S.E.M. for two separate experiments was $183 \pm 36$ mu. (*cf.* assay 5-e with assays 16-b and 23, Table 2; $P < 0.05$).

*Blood ACTH in stressed rats.* (1) *Ether anesthesia.* Exposure to ether for 45 seconds produced a barely detectable level of ACTH in blood of intact rats (Table 1 and Appendix I, assay 3-b); the concentration was estimated to be $0.9 \pm 0.5$ mu. ACTH/100 ml. (P test of significance from zero, $= 0.05$ to 0.1). A striking increase in the concentration of blood ACTH occurred in adrenalectomized rats similarly stressed (cf. assay 5-a with 5-b, Table 1). Within 2 minutes after the beginning of ether anesthesia the blood ACTH titer had increased from $1.6 \pm 0.7$ to $9.5 \pm 2.8$ mu./100 ml.

(2) *Ether plus scald.* One minute after scald (2 minutes after initial exposure to ether) the blood of the intact rat contained a small, but detectable, quantity of ACTH (Table 1 and Appendix I, assay 4-b, $P = 0.02$).

TABLE 1. BLOOD ACTH

| Stress employed | Duration of stress | Experimental animal | Blood ACTH mu* per 100 ml. M. ±S.E.M. | Assay No. |
|---|---|---|---|---|
| None | | Intact | <0.5 | 1-a |
| None | | Intact | <0.5 | 2 |
| None | | Intact | <0.5 | 3-a |
| None | | Intact | <0.5 | 4-a |
| None | | Adrenx—1 wk. | 1.6 ± 0.7 | 5-a |
| None | | Adrenx—1 wk. | 3.5 ± 1.0 | 6-a |
| None | | Adrenx—1 wk. | 3.5 ± 0.6 | 7-a |
| 45 sec. exposure to ether; decapitated 2 min. after application of stress stimulus | 45 sec. | Intact | 0.9 ± 0.5 | 3-b |
| | | Adrenx—1 wk. | 9.5 ± 2.8 | 5-b |
| 2 min. exposure to ether; decapitated 2 min. after application of stress stimulus | 2 min. | Adrenx—1 wk. | 4 to 8† | 8-a |
| 45 sec. exposure to ether followed by a 7-sec. scald; decapitated 2 min. after application of stress stimulus | 2 min. | Intact | 1.1 ± 0.4 | 4-b |
| | | Adrenx—1 wk. | 4 to 8† | 5-c |
| 45 sec. exposure to ether followed by a 7-sec. scald; decapitated 6 min. after application of stress stimulus | 6 min. | Intact | <0.5 | 9-a |
| | | Adrenx—1 wk. | 7.1 ± 1.5 | 6-b |
| 45 sec. exposure to ether followed by a 7-sec. scald; decapitated 24 ± 4 hr. after application of stress stimulus | 24 ± 4 hr. | Intact | <0.5 | 9-b |
| Continuous ether anesthesia plus rapid exsanguination from aorta | 5.5 min. | Intact | 2.8 ± 0.6 | 10 |
| | 4.5 min. | Intact | 2.1 ± 0.6 | 11-a |
| | 4.5 min. | Intact | 2.9 ± 0.8 | 11-b |
| | 2.5 min. | Adrenx—1 wk. | 10.9 ± 4.1 | 8-b |
| | 4.5 min. | Adrenx—1 wk. | 12.0 ± 2.5 | 12 |
| | 6 min. | Adrenx—1 wk. | 11.0 ± 2.0 | 13 |
| | 5 min. | Adrenx—1 wk. | 15.4 ± 2.6 | 14 |
| | 3 min. | Adrenx—2 wk. | 13.7 ± 2.3 | 15 |
| | 15 min. | Adrenx—2½ wk. | 16.5 ± 2.5 | 16-a |
| Continuous ether anesthesia plus repeated bleeding—bled from aorta during the 3rd, 6th and 11th min. of anesthesia; blood volume not restored | 3 min. | Intact | 2 to 6† | 17 |
| | 6 min. | | 2 to 6† | |
| | 11 min. | | >8† | |
| Continuous ether anesthesia plus repeated bleeding—bled from aorta during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume not restored | 3 min. | Adrenx—2 wk. | 15.3 ± 3.0 | 18-a |
| | 7 min. | | 16.5 ± 4.4 | |
| | 11 min. | | 33.9 ± 9.0 | |
| | 15 min. | | 53.5 ±25.6 | |
| Continuous ether anesthesia plus repeated bleeding—bled from aorta during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume restored | 3 min. | Adrenx—2 wk. | 12.1 ± 1.5 | 19-a |
| | 7 min. | | 10.7 ± 1.5 | |
| | 11 min. | | 15.8 ± 1.8 | |
| | 15 min. | | 11.0 ± 1.8 | |

* One milliunit (mu.) equals one-thousandth of a U.S.P. unit of ACTH (La-1-A).
† Estimate of concentration based on single-dose assays.

However, the additional stress imposed by scald did not produce a significant increase in the concentration over that in rats exposed to ether alone. In the group decapitated 5 minutes after scald (6 minutes after exposure to ether) and in the group decapitated 20 ± 4 hours after scald, the con-

centration of ACTH in blood was too low to be detected by the method of assay (Table 1 and Appendix I, assays 9-a and 9-b). As in the intact rat, ether plus scald produced an increase in the concentration of blood ACTH in adrenalectomized rats over the level observed in the non-stressed animal (*cf.* assays 6-a and 6-b, Table 1). The concentration of blood ACTH, $7.5 \pm 1.5$ mu./100 ml., in adrenalectomized rats decapitated 5 minutes post-scald was not significantly different from that in rats exposed to ether alone.

(3) *Ether plus rapid exsanguination.* Ether plus rapid exsanguination in the intact rat induced a significant increase in the concentration of blood ACTH over the level observed in the group exposed to ether alone ($P < 0.01$). The weighted M. $\pm$ S.E.M. of the analyses of three separate experiments is $2.6 \pm 0.3$ mu./100 ml. blood (Table 1 and Appendix I, assays 10, 11-a and 11-b). This same stress applied to the adrenalectomized rat one week post-operative induced a striking increase in concentration of blood ACTH over non-stress levels. The weighted M. $\pm$ S.E.M. for three separate experiments is $12.1 \pm 1.4$ mu. (Table 1 and Appendix II, assays 12, 13 and 14). In contrast to the intact rat, the combination of continuous ether anesthesia and rapid exsanguination in the adrenalectomized animal did not induce a significantly higher blood ACTH titer than that induced by ether alone (Table 1 and Appendix II, assays 8-a and 8-b; $P = 0.4$). The concentration of ACTH in blood of rats 2 weeks after adrenalectomy did not differ from that of rats one week post-adrenalectomy under these experimental conditions (Table 1 and Appendix II, assays 12, 13, 14, 15 and 16-a).

(4) *Ether plus repeated bleeding.* Samples of blood collected from the abdominal aorta of the intact rat during the third, sixth, and eleventh minute of an 11-minute exposure to ether were analyzed for ACTH. Quantitative estimates are not available since single-dose assays were performed (Table 1 and Appendix I, assay 17). A dose of eluate equivalent to 12 ml. blood induced a significant degree of adrenal ascorbic acid depletion. The data suggest that blood obtained during the third and the sixth minute contains 2 to 4 mu. ACTH/100 ml. A dose of eluate equivalent to 12 ml. blood obtained during the eleventh minute induced an average adrenal ascorbic acid depletion greater than that induced by 1.0 mu. ACTH Standard; the results suggest that this pool of blood contained more than 8 mu. ACTH/100 ml.

Quantitative data are available for samples of blood collected from adrenalectomized rats during the third, the seventh, the eleventh, and the fifteenth minute of ether anesthesia. Animals infused with 0.9% NaCl solution showed a marked increase in blood-ACTH over control levels when bled during the third minute of exposure to ether. This high level

was maintained during the other bleeding periods (Table 1, assay 19-a). In the animals bled repeatedly, but whose blood volume was not replaced, the same order of increase in the blood-ACTH titer occurred during the third and the seventh minute (Table 1, assay 18-a). Apparently higher concentrations existed during the eleventh and the fifteenth minute. The assay data (Appendix II, assay 18-a) indicate that the dose of blood injected were too high to be included within the linear part of the log-dose response curve; furthermore, the variances of the assays are great and the difference between the means for the eleventh and the fifteenth-minute samples on the one hand, and for the third and the seventh-minute samples on the other, are not statistically significant. It is to be noted that the blood ACTH titers, both in the intact and in the adrenalectomized rat, remained fairly constant until circulating blood volume had been markedly reduced. The reduction in circulating blood volume could account for the increase in blood ACTH titer under circumstances in which rate of discharge is constant.

*Pituitary ACTH in stressed rats.* Single dose assays indicate that the content of ACTH in the pituitary of the stressed intact rat was 50 to 150 mu. (Table 2 and Appendix III, assays 3-d and 4-d) and that of the stressed adrenalectomized rat one week post-operative is 100 to 200 mu. (Table 2 and Appendix III, assays 5-f and 5-g). Multidose assays on pituitaries from intact rats or from rats sacrificed 2 weeks after adrenalectomy indicate that 15 minutes exposure to ether plus repeated bleeding, the most severe stresses employed in the present study, did not induce a significant decrease in pituitary stores of ACTH (Table 2 and Appendix III, assays 18-b, 19-b and 20). However, the stresses employed may have produced a net loss of ACTH from the pituitary which could not be detected by the assay method.

## DISCUSSION

Three mechanisms have been proposed for the regulation of the rate of discharge of ACTH from the adenohypophysis:

(1) *Cortical hormone-titer thesis.* The evidence for a regulatory influence of adrenocorticosteroids on pituitary ACTH activity has been reviewed by Sayers (1950) and by Harris (1951). There appears to be little doubt that these steroids inhibit release of ACTH. The precise mechanism of action is unknown and it has not been established whether the adrenocorticosteroids act directly on the adenohypophysis or indirectly through the mediation of the hypothalamus. Furthermore, it has not been established whether the inhibitory effect is mediated by the adrenocorticosteroids themselves or some unidentified consequence of their action. The cortical hormone-titer thesis holds that application of a stressful stimulus induces an increased

TABLE 2. PITUITARY ACTH

| Stress employed | Duration of stress | Experimental animal | ACTH content mu.* per pituitary M. ±S.E.M. | Assay No. |
|---|---|---|---|---|
| None | | Intact | 64.4±13.8 | 21 |
| None | | Intact | 80.1±11.0 | 22 |
| None | | Intact | 50 to 100† | 1-b |
| None | | Intact | 50 to 100† | 3-c |
| None | | Intact | 50 to 100† | 4-c |
| None | | Adrenx—1 wk. | 85.0±30.0 | 5-e |
| None | | Adrenx—2 wk. | 161.0±79.0 | 23 |
| None | | Adrenx—2 wk. | 189.6±41.1 | 16-b |
| 45 sec. exposure to ether; decapitated 2 min. after application of stress stimulus | 45 sec. | Intact | 50 to 100† | 3-d |
| | | Adrenx—1 wk. | 100 to 200† | 5-f |
| 45 sec. exposure to ether followed by a 7-sec. scald; decapitated 2 min. after application of stress stimulus | 2 min. | Intact | 50 to 150† | 4-d |
| | | Adrenx—1 wk. | 100 to 200† | 5-g |
| Continuous ether anesthesia plus rapid exsanguination from aorta | 15 min. | Adrenx—2 wk. | 171.8±30.4 | 24 |
| | | Adrenx—2½ wk. | 158.8±16.6 | 16-c |
| Continuous ether anesthesia plus repeated bleeding; bled from aorta during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume not restored | 15 min. | Intact | 75.4±15.8 | 20 |
| | | Adrenx—2 wk. | 116.8±25.1 | 18-b |
| Continuous ether anesthesia plus repeated bleeding; bled from aorta during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume restored | 15 min. | Adrenx—2 wk. | 157.4±33.7 | 19-b |

* One milliunit (mu.) equals one-thousandth of a U.S.P. unit of ACTH (La-1-A).
† Estimate of pituitary ACTH content based on single-dose assays.

rate of "utilization" of adrenocorticosteroids, thereby effecting a reduction in blood steroid titer; the decrease in steroid titer is the stimulus for accelerated discharge of ACTH from the adenohypophysis. No conclusive evidence has been presented regarding rate of "utilization" of adrenocortical steroids by tissues; therefore, the role of adrenocorticosteroid titer in the period immediately following the application of a stressful stimulus must remain uncertain.

(2) *Central neural mechanism.* Whereas the cortical hormone-titer thesis emphasizes the importance of the action of the stimulus on the periphery of the organism, the neural concept emphasizes the role of the hypothalamus in initiating a series of events which results in increased activity of the adrenal cortex. Neither the manner in which the stimulus "fires" the hypothalamus nor the mechanism by which the hypothalamus influences the adenohypophysis to discharge ACTH has been established. According to Harris and his associates (Green and Harris, 1947, and Harris, 1952), the activation of certain hypothalamic nuclei causes the release of a neurohumor (s) which is transmitted by way of the hypothalamic-hypo-

physeal portal system to the adenohypophysis where it effects the release of ACTH. The nature of the neurohumor(s) has not been established.

(3) *Epinephrine theory.* It has been suggested (McDermott *et al.*, 1950; Long, 1952) that the chain of events which follows the application of a stress stimulus includes the following links: (1) hypothalamic stimulation, (2) transmission of impulses via the brain stem to the adrenal medulla to stimulate release of epinephrine, (3) humoral transmission of epinephrine to the adenohypophysis, and (4) direct action of epinephrine on adenohypophyseal cells to induce release of ACTH. The *epinephrine theory* is difficult to reconcile with the following facts: (1) Epinephrine is a relatively weak stimulus of the pituitary-adrenal system (PAS) (Coutinho *et al.*, 1953), (2) adrenal-denervated (Vogt, 1947; Colfer *et al.*, 1950), adrenal-medullectomized (Gordon, 1950; Vogt, 1952), and completely sympathectomized animals (Hume and Wittenstein, 1950; Recant *et al.*, 1950; Hume, 1952) exhibit little or no inertia of the PAS.

In summary, the available evidence suggests that regulation of ACTH involves both a humoral mechanism (blood-titer of adrenocortical hormone) and a neural mechanism (hypothalamus). The relative importance of these two has not been established; the nature and the duration of the PAS stimulus may determine the extent to which one mechanism is called into play as compared to the other. The immediate acceleration in ACTH release which follows application of a stressful stimulus may be called forth by the neural mechanism, the subsequent rate of discharge may be regulated by the titer of adrenocorticosteroids.

The theories of pituitary ACTH regulation are based on experiments which have utilized indirect indices of ACTH discharge in intact animals: eosinopenia, lymphocytopenia, thymic involution, increased excretion of urinary steroids, and adrenal ascorbic acid depletion. The end-points of the responses have been measured from one to six hours after application of the stress stimulus. These indices reflect changes due to accelerated adrenocortical activity; they do not provide precise information regarding the rate of release of pituitary ACTH. The development of methods for the concentration of ACTH in blood makes it possible to estimate rate of discharge of ACTH in animals, both intact and adrenalectomized, under a wide variety of experimental conditions. Information regarding the dynamic nature of the mechanism or mechanisms which regulate pituitary ACTH activity now can be obtained.

The results of the present study indicate that the concentration of ACTH in the blood of the intact non-stressed rat is extremely small, i.e., less than 0.5 mu./100 ml. These data indicate that the adenohypophysis is relatively inactive under optimal environmental conditions; they further suggest that the adrenal cortex requires relatively minute quantities of

circulating ACTH for maintenance of adrenocortical function under these conditions.

One week after adrenalectomy the blood ACTH concentration has increased from nondetectable levels to 2.6 mu./100 ml. The increase in ACTH titer occurs in the absence of stressful stimuli. This may be interpreted to mean that the reduction in concentration of adrenocorticosteroids accelerates the rate of release of ACTH. The significantly higher ACTH content of the pituitary in the rat after adrenalectomy indicates that increased synthesis as well as increased secretion of the hormone occurs following prolonged reduction of circulating adrenocorticosteroids.

The stresses employed in this study induce a small, but significant, increase in the concentration of ACTH in the blood of the intact rat. The dynamic character of the mechanism(s) involved is illustrated by the fact that the increase is observed within 2 minutes after application of a stressful stimulus. The observations are in agreement with those of Gray and Munson (1951).

The changes in concentration of blood ACTH following scald are of some interest. Although the data are limited and no definitive conclusions can be established, they may be interpreted to mean that a neural mechanism (hypothalamic factor?) activates the adenohypophysis to discharge ACTH at an accelerated rate. The initially high blood ACTH titer, in turn, stimulates secretory activity of the adrenal cortex. The reduced titer observed at five minutes and at 24 hours after scald may be a consequence of the inhibitory influence of blood adrenocorticosteroids. Inasmuch as the blood ACTH titer immediately after ether plus scald is not significantly different from that of rats exposed to ether alone, two other possible explanations must be considered: (1) The intensity of the scald stress is not of sufficient magnitude to induce prolonged ACTH discharge. (2) The concentration of ACTH in blood, though less than 0.5 mu./100 ml. may be higher than that which exists in the non-stressed animal and great enough to induce accelerated adrenocortical discharge. The second possibility would appear to be the most likely explanation. However, improvement in the technic for analysis of blood ACTH must be made before the problem is finally solved.

In the experiments involving ether plus laparotomy plus exsanguination, the blood ACTH titer is significantly higher than that observed in intact rats after scald or after ether alone. Furthermore, the single-dose assays on blood from intact rats subjected to repeated bleeding suggest that ACTH concentrations increase with progressive blood loss. This is in contrast to scald, the effect of which was discussed above. The data suggest that certain types of severe stress, *i.e.*, those that threaten the life of the organism, may induce a marked increase in the rate of release of ACTH.

A significant increase in the concentration of blood ACTH occurred after application of stress to the adrenalectomized rat. However, no data presented in this report (other than those concerned with repeated bleeding associated with progressive reduction in blood volume) indicate that the continued application of a stress stimulus further accelerates discharge of ACTH in the adrenalectomized animal. There is no significant difference between the ACTH concentration of blood obtained from rats exposed to ether for 45 seconds and those stressed by ether plus laparotomy plus exsanguination, either for 3 minutes or for 15 minutes. Unlike the intact animal, the blood ACTH titer remains high at 5 minutes after scald. The striking difference between the intact and the adrenalectomized rat in response to stress clearly emphasizes the dominant role of adrenocorticosteroids in pituitary ACTH regulation. The influence of a neural mechanism may be fully manifested only in the absence of adrenocorticosteroids.

The experimental data on blood ACTH in intact and adrenalectomized rats offer additional evidence in support of the thesis that the titer of adrenocorticosteroids regulates pituitary ACTH activity. If it can be ascertained that no functional accessory adrenocortical tissue exists in adrenalectomized animals, the data also offer convincing evidence that a factor other than the concentration of adrenocorticosteroids in blood influences the rate of discharge of ACTH from the adenohypophysis. This factor may involve the hypothalamus. Experiments are now in progress to determine the rate of discharge of ACTH from the pituitary of adrenalectomized rats with lesions in the hypothalamus. If the adrenalectomized hypothalamic-lesioned rat does not respond with an increase in blood ACTH titer after application of a stress, this preparation should prove of value in testing "neurohumors" extracted from the hypothalamus for their ability to induce release of ACTH.

## SUMMARY

The concentration of ACTH in the blood of the non-stressed intact rat is too low to be detected by the technics employed and is estimated to be less than 0.5 milliunit per 100 ml. One week after adrenalectomy the concentration has increased to 3.2 milliunits per 100 ml.

Two minutes after the application of a stressful stimulus (exposure to ether for 45 seconds or exposure to ether plus a standardized scald), ACTH is detectable in blood of the intact rat. The mean concentration is estimated to be 1 milliunit per 100 ml. These same stresses applied to the adrenalectomized rat induce a two to threefold increase in blood ACTH over non-stress levels.

Six minutes after exposure to ether plus a standardized scald, ACTH is

not detectable in the blood of the intact rat, but is present in high concentration in the adrenalectomized animal.

Continuous exposure to ether plus exsanguination induces a significant increase in the blood-ACTH titer of the intact rat. The mean concentration of ACTH is estimated to be 2.6 milliunits per 100 ml. The blood-ACTH titer of the adrenalectomized rat similarly stressed is not significantly different from that of the adrenalectomized rat exposed to ether alone.

It is concluded that: (1) Accelerated release of ACTH occurs immediately after application of stressful stimuli. (2) The concentration of adrenocorticosteroids in blood and/or tissues exerts the dominant role in regulating pituitary ACTH. (3) A hypothalamic factor may initiate pituitary discharge of ACTH following the application of stressful stimuli.

The ACTH content of pituitary obtained from the non-stressed or from the stressed intact rat is approximately 70 milliunits. Two weeks after adrenalectomy the mean content has increased to 170 milliunits. There is no significant difference between the ACTH content of pituitary obtained from non-stressed adrenalectomized and from stressed adrenalectomized animals. However, the increased rate of discharge which follows stress may have resulted in a decrease in pituitary stores which could not be detected by the assay technic.

The mechanisms concerned with the regulation of pituitary ACTH are discussed.

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(*Continued on page 636*)

APPENDIX I. BLOOD ACTH IN INTACT RATS

| Assay No. | Experiment | Duration of stress | Number of donor rats | Dose per 100 g. assay rat | Ascorbic acid depletion mg./100 g. fresh adrenal individual responses | Avg. | Concentration mu.*/100 ml. blood M. ± S.E.M. |
|---|---|---|---|---|---|---|---|
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.25 | 215, 116, 128, 196<br>111, 158, 73, 51, 52; 78, 86 | 164<br>87 | |
| 1-a | Decapitated; no stress | None | 48 | 18 ml. | 56, 15, 5 | 22 | <0.5+ |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 124, 83, 91, 143, 146, 135<br>121, 81, 85, 108, 119, 130<br>23, 5, 28, 139 | 120<br>107<br>39 | |
| 2 | Decapitated; no stress | None | 48 | 18 ml. | 21, −5, 8 | 8 | <0.5+ |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 57, 118, 46, 84, 45, 113<br>14, 93, 81, 75, 24, 49<br>6, 40, 74, 46, 27, 22 | 77<br>56<br>36 | |
| 3-a | Decapitated; no stress | None | 76 | 42 ml./rat<br>21 | −8, 10, 21, 29, −4, −4<br>−10, 11, 5, 24, 16, 0 | 7<br>8 | <0.5 |
| 3-b | 45 sec. exposure to ether; decapitated 2 min. after application of stress stimulus | 2 min. | 55 | 32 ml./rat<br>16 | 7, 65, 61<br>−6, 11, 36, 50, 19 | 44<br>22 | 0.9±0.45 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 140, 97, 105, 155, 114, 119<br>44, 80, 99, 105, 83, 116, 110<br>27, 20, 35, 92, 50 | 122<br>91<br>45 | |
| 4-a | Decapitated; no stress | None | 76 | 36 ml. | −1, 3, 2, 93 | 24 | <0.5 |
| 4-b | 45 sec. exposure to ether followed by 7-sec. scald; decapitated 2 min. after application of stress stimulus | 2 min. | 31 | 18 ml.<br>9 | 52, 33, 47<br>1, 63, 7, −9 | 44<br>16 | 1.1±0.4 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 135, 96, 113, 166, 126, 59, 101<br>86, 90, 117, 97, 98, 42<br>89, 28, 63, 72, 32, 40 | 114<br>83<br>54 | |
| 9-a | 45 sec. exposure to ether followed by 7-sec. scald; decapitated 6 min. after application of stress stimulus | 6 min. | 50 | 24 ml. | −4, 65<br>36, −8, −7, 41 | 31<br>16 | <0.5 |
| 9-b | 45 sec. exposure to ether followed by 7-sec. scald; decapitated 24±4 hr. after application of stress stimulus | 24±4 hr. | 83 | 24 ml. | 31, 37, 15, 20 | 26 | <0.5 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.25 | 196, 216, 202, 91<br>38, 38, 34, 45 | 176<br>39 | |
| 10 | Continuous ether anesthesia plus rapid exsanguination from aorta | 5½ min. | 34 | 12 ml.<br>6<br>3 | 49, 51, 108, 47<br>19, 19, 43, 34, 43<br>18, 11, 33, −10 | 61<br>34<br>11 | 2.8±0.6 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.25 | 68, 163, 159, 98, 176<br>38, 87, 46, 47, 62, 56 | 133<br>56 | |
| 11-a | Continuous ether anesthesia plus rapid exsanguination from aorta | 4½ min. | 17 | 12 ml.<br>6 | 60, 60, 67, 56, 58<br>19, 17, 1, 16, −12 | 60<br>7 | 2.1±0.5 |
| 11-b | Continuous ether anesthesia plus rapid exsanguination from aorta | 4½ min. | 19 | 12 ml.<br>6 | 108, 82, 86, 52<br>15, 43, 47, 3, 15 | 81<br>25 | 2.9±0.8 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 57, 64, 82, 82, 70, 35, 82<br>59, 91, 31, 16, 5<br>25, −6, 21, 26, −2 | 61<br>40<br>13 | |
| 17 | Continuous ether anesthesia plus repeated bleeding; bled from aorta during the 3rd, 6th, and 11th min. of anesthesia. Blood volume not restored | 3 min.<br>6 min.<br>11 min. | 43 | 12 ml.<br>12 ml.<br>12 ml. | 59, 42, 80, 5, 66<br>60, 72, 32, 45, 67<br>98, 136, 107, 29 | 50<br>56<br>93 | 2 to 6†<br>2 to 6†<br>>8† |

* One milliunit (mu.) equals one-thousandth of a U.S.P. unit of ACTH (La-1-A).
† Estimate of concentration based on single-dose assay.

APPENDIX II. BLOOD ACTH IN ADRENALECTOMIZED RATS

| Assay No. | Experiment | Duration of stress | Number of donor rats | Dose per 100 g. assay rat | Ascorbic acid depletion mg./100 g. fresh adrenal individual responses | Avg. | Concentration mu.*/100 ml. blood M. ± S.E.M. |
|---|---|---|---|---|---|---|---|
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 131, 79, 129, 110, 123, 111, 126<br>79, 20, 108, 139, 95, 130, 85<br>83, 30, 92, 54, 66, 68, 58 | 122<br>94<br>64 | |
| 5-a | Adrenx *1 wk.*: decapitated without stress | None | 24 | 12 ml.<br>6 | 67, 36, 117, 49<br>11, 11, −24, 14 | 67<br>3 | 1.6 ± 0.65 |
| 5-b | Adrenx *1 wk.*: 45 sec. exposure to ether; decapitated 2 min. after application of stress stimulus | 45 sec. | 25 | 12 ml.<br>6 | 137, 126, 148, 120<br>79, 73, 125, 74 | 133<br>88 | 9.5 ± 2.75 |
| 5-c | Adrenx *1 wk.*: 45 sec. exposure to ether followed by 7-sec. scald; decapitated 2 min. after application of stress stimulus | 2 min. | 28 | 12 ml.<br>6 | 83, 94, 111<br>78, 76, 150 | 96<br>101 | 4 to 8† |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 148, 201, 133, 215, 131<br>113, 89, 47, 83<br>102, 105, 49, 10, 79 | 166<br>83<br>69 | |
| 6-a | Adrenx *1 wk.*: decapitated without stress | None | 28 | 11.2 ml.<br>5.6<br>2.8 | 62, 105, 123, 35, 140<br>27, 28, 49, 48, 32, 45, 69<br>38, 21, 32, 53, 25, −8, 24 | 93<br>43<br>26 | 3.5 ± 0.95 |
| 6-b | Adrenx *1 wk.*: 45 sec. exposure to ether followed by a 7-sec. scald; decapitated 6 min. after application of stress stimulus | 6 min. | 47 | 11.2 ml.<br>5.6<br>2.8 | 145, 139, 165, 111, 101<br>40, 114, 115, 101, 99, 16, 80<br>85, 84, 77, 19, 47, 67, 39, 10, 54 | 132<br>92<br>54 | 7.1 ± 1.5 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 132, 134, 125, 200, 153, 175<br>49, 90, 79, 81, 85<br>21, 27, 27, 102, −2 | 153<br>77<br>34 | |
| 7-a | Adrenx *1 wk.*: decapitated without stress | None | 20 | 12 ml.<br>6 | 64, 33, 72, 85, 9<br>72, 22, 17 | 53<br>37 | 3.5 ± 0.6 |
| 7-b | Adrenx *1 wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 5 min. | 10 | 6 ml.<br>3<br>1.5 | 29, 83, 86, 122, 55, 70<br>−2, 35, 82, 6<br>−34, 13, 26 | 74<br>30<br>2 | 7.1 ± 1.4 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 159, 124, 165<br>116, 158, 99, 126, 87<br>58, 74, 50, 62 | 152<br>116<br>61 | |
| 8-a | Adrenx *1 wk.*: decapitated after exposure to ether for 2 min. | 2 min. | 19 | 12 ml.<br>6 | 88, 85, 53<br>114, 53, 59, 76 | 75<br>61 | 4 to 8† |
| 8-b | Adrenx *1 wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 2½ min. | 18 | 12 ml.<br>6 | 157, 148, 155<br>115, 122, 70, 194 | 154<br>128 | 10.9 ± 4.1 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 148, 204, 128, 194, 157, 207<br>47, 135, 182, 129, 170, 93<br>6, 27, 20, 45, 88 | 173<br>126<br>37 | |
| 12 | Adrenx *1 wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 6 min. | 28 | 6 ml.<br>3<br>1.5 | 177, 148, 166, 186<br>54, 97, 72, 67, 129<br>12, 12, 57, −36, 30, 47, 23 | 169<br>84<br>21 | 12.0 ± 2.5 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 195, 118, 145, 112, 140, 110<br>71, 123, 60, 82, 149<br>25, 19, 35, 25, 33 | 137<br>97<br>27 | |
| 13 | Adrenx *1 wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 6 min. | 40 | 6 ml.<br>3 | 72, 124, 56, 140<br>105, 59, 70, 73, 21 | 98<br>66 | 11.0 ± 2.0 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 125, 144, 100, 151, 140, 113, 98, 143<br>82, 40, 85, 44, 116, 112<br>46, 69, 29, 56, 23, 5, −28 | 127<br>80<br>27 | |
| 14 | Adrenx *1 wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 5 min. | 10 | 6 ml.<br>3<br>1.5 | 113, 126, 130, 31<br>115, 63, 90, 90<br>42, 18, 53, −1, 43 | 100<br>90<br>31 | 15.4 ± 2.6 |

* One milliunit (mu.) equals one-thousandth of a U.S.P. unit of ACTH (La-1-A).
† Estimate of potency based on single-dose assays.

APPENDIX II—*Continued*

| Assay No. | Experiment | Duration of stress | Number of donor rats | Dose per 100 g. assay rat | Ascorbic acid depletion mg./100 g. fresh adrenal individual responses | Avg. | Concentration mu.*/100 ml. blood M. ± S.E.M. |
|---|---|---|---|---|---|---|---|
| | U.S.P. Standard ACTH | | | 1.0 mu. | 140, 122, 171, 102, 122, 122, 136 | 131 | |
| | | | | 0.5 | 95, 64, 89, 80, 129, 127, 128 | 102 | |
| | | | | 0.25 | 49, 56, 65, 111, 105, 71 | 76 | |
| 15 | Adrenx *2 wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 3 min. | 76 | 6 ml. | 105, 103, 222, 165, 128 | 145 | 13.7 ± 3.0 |
| | | | | 3 | 44, 121, 138, 40, 52, 80 | 83 | |
| | | | | 1.5 | 83, 106, 50, 55, 32, 61, 20 | 61 | |
| | U.S.P. Standard ACTH | | | 1.0 mu. | 109, 103, 106, 125, 112 | 112 | |
| | | | | 0.5 | 66, 59, 66, 69, 51 | 64 | |
| | | | | 0.25 | 71, 40, 48, 31, 25 | 43 | |
| 16-a | Adrenx *2½ wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 15 min. | 12 | 6 ml. | 91, 121, 128, 88 | 107 | 16.5 ± 2.5 |
| | | | | 3 | 72, 65, 80, 107, 23, 42 | 65 | |
| | | | | 1.5 | 35, 57, 0, 82, 62 | 47 | |
| | U.S.P. Standard ACTH | | | 1.0 mu. | 112, 149, 99, 81, 180 | 124 | |
| | | | | 0.5 | 115, 88, 64, 105 | 93 | |
| | | | | 0.25 | 98, 57, −2, 20, 20 | 39 | |
| 18-a | Adrenx *2 wk.*: continuous ether anesthesia plus repeated bleeding from aorta; bled during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume not restored | 3 min. | 45 | 6 ml. | 55, 167, 110, 117, 153 | 120 | 15.3 ± 3.3 |
| | | | | 3 | 68, 89, 37, 131 | 81 | |
| | | | | 1.5 | 69, 19, 9, 49, 40 | 39 | |
| | | 7 min. | | 6 ml. | 67, 171 | 119 | 16.5 ± 4.4 |
| | | | | 3 | 100, 63, 46, 84 | 73 | |
| | | | | 1.5 | 70, 56, 73, 38 | 59 | |
| | | 11 min. | | 6 ml. | 181, 171, 154 | 169 | 33.9 ± 9.0 |
| | | | | 3 | 145, 132, 82, 150, 116 | 125 | |
| | | | | 1.5 | 70, 56, 73, 38 | 92 | |
| | | 15 min. | | 6 ml. | 187, 102, 209 | 166 | 53.5 ± 25.6 |
| | | | | 1.5 | 125, 109, 130 | 122 | |
| | U.S.P. Standard ACTH | | | 1.0 mu. | 145, 171, 155, 173, 110, 143 | 150 | |
| | | | | 0.5 | 44, 118, 128, 68, 82 | 88 | |
| | | | | 0.25 | 4, −8, 59, 12, 39 | 21 | |
| 19-a | Adrenx *2 wk.*: continuous ether anesthesia plus repeated bleeding from aorta; bled during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume restored | 3 min. | 60 | 6 ml. | 128, 66, 84, 120, 94 | 98 | 12.1 ± 1.5 |
| | | | | 3 | 73, 132, 16, 84, 30 | 78 | |
| | | | | 1.5 | 28, 58, −19, 34, 35 | 27 | |
| | | 7 min. | | 6 ml. | 65, 111, 92, 72, 126 | 94 | 10.7 ± 1.5 |
| | | | | 3 | 35, 17, 87, 39, 64 | 48 | |
| | | | | 1.5 | 17, 42, 27, 19, 51 | 31 | |
| | | 11 min. | | 6 ml. | 108, 104, 170, 151, 152 | 137 | 15.8 ± 1.8 |
| | | | | 3 | 51, 108, 89, 58, 96 | 80 | |
| | | | | 1.5 | −1, 30, 75, 40, 45 | 38 | |
| | | 15 min. | | 6 ml. | 87, 90, 115, 99, 128 | 104 | 11 ± 1.8 |
| | | | | 3 | 88, 56, 80, 117, 84, 80 | 84 | |
| | | | | 1.5 | 32, 22, 9, −5, 40 | 20 | |
| | U.S.P. Standard ACTH | | | 1.0 mu. | 150, 126, 91, 180, 126, 196 | 145 | |
| | | | | 0.5 | 111, 87, 135, 109, 95 | 107 | |
| | | | | 0.25 | 80, 42, 44, 51, 101, 3, 56 | 54 | |

## Appendix III. Rat pituitary ACTH

| Assay No. | Experiment | Duration of stress | Number of donor rats | Dose per 100 g. assay rat | Ascorbic acid depletion mg./100 g. fresh adrenal individual responses | Avg. | Content mu.* per pituitary M.±S.E.M. |
|---|---|---|---|---|---|---|---|
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.25 | 215, 281, 116, 128, 111, 196<br>111, 158, 73, 51, 52, 78, 86 | 175<br>87 | |
| 1-b | Intact: decapitated without stress | None | 48 | 0.01 pituitary | 201, 157, 98, 123, 100, 79 | 126 | 50 to 100† |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 140, 97, 105, 155, 114, 119<br>44, 80, 99, 105, 83, 116, 110<br>27, 20, 35, 92, 50 | 122<br>91<br>45 | |
| 3-c | Intact: decapitated without stress | None | 48 | 0.01 pituitary | 77, 40, 106, 117, 125, 103 | 95 | 50 to 100† |
| 3-d | Intact: 45 sec. exposure to ether; decapitated 2 min. after application of stress stimulus | 45 sec. | 55 | 0.01 pituitary | 92, 97, 94, 135, 135 | 111 | 50 to 150† |
| 4-d | Intact: 45 sec. exposure to ether followed by a 7-sec. scald; decapitated 2 min. after application of stress stimulus | 2 min. | 76 | 0.01 pituitary | 147, 79, 123, 118, 120, 155 | 124 | 50 to 150† |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 93, 128, 123, 122, 113<br>122, 95, 63, 115, 96<br>108, 73, 79, 45, 54 | 116<br>98<br>72 | |
| 4-e | Intact: decapitated without stress | None | 76 | 0.01 pituitary<br>0.005 pituitary | 109, 72, 109, 59<br>65, 47, 106, 84 | 87<br>76 | 50 to 100† |
| 5-e | Adrenx *1 wk.*: decapitated without stress | None | 24 | 0.005 pituitary<br>0.0025 pituitary | 125, 121, 60<br>97, 51, 47, 57 | 102<br>63 | 85±30 |
| 5-f | Adrenx *1 wk.*: 45 sec. exposure to ether; decapitated 2 min. after application of stress stimulus | 45 sec. | 25 | 0.005 pituitary<br>0.0025 pituitary | 106, 114, 112, 108<br>88, 112, 115, 139 | 110<br>114 | 100 to 200† |
| 5-g | Adrenx *1 wk.*: 45 sec. exposure to ether followed by 7-sec. scald; decapitated 2 min. after application of stress stimulus | 2 min. | 28 | 0.005 pituitary<br>0.0025 pituitary | 143, 112, 88, 85<br>88, 107, 114, 96 | 107<br>101 | 100 to 200† |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 94, 101, 140, 58, 145<br>137, 99, 68, 59<br>43, 19, 30, 54, 16, 53 | 110<br>91<br>36 | |
| 16-b | Adrenx *2½ wk.*: decapitated without stress | None | 6 | 0.005 pituitary<br>0.0025 pituitary<br>0.00125 pituitary | 117, 102, 114, 159<br>20, 105, 70, 43<br>85, 5, 51, 13 | 123<br>60<br>39 | 189.6±41.1 |
| 16-c | Adrenx *2½ wk.*: continuous ether anesthesia plus rapid exsanguination from aorta | 15 min. | 6 | 0.005 pituitary<br>0.0025 pituitary<br>0.00125 pituitary | 110, 49, 135, 136, 86<br>68, 31, 23, 47<br>9, 58, 22, 36, 54 | 103<br>42<br>36 | 158.8±16.6 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 110, 127, 170, 54, 111<br>105, 57, 59, 131, 126<br>36, 33, 2, 49, 101 | 115<br>96<br>44 | |
| 18-b | Adrenx *2 wk.*: continuous ether anesthesia plus repeated bleeding; bled from aorta during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume not restored | 15 min. | 45 | 0.005 pituitary<br>0.0025 pituitary<br>0.00125 pituitary | 57, 122, 136, 98, 154<br>36, 24, 65, 51, 6<br>−13, 20, −44, 29, 81 | 113<br>34<br>19 | 116.8±25.1 |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5<br>0.25 | 125, 82, 213, 156, 147, 168<br>79, 23, 62, 62, 82, 85<br>49, 38, 12, 51, 88, 61 | 149<br>66<br>51 | |
| 19-b | Adrenx *2 wk*: continuous ether anesthesia plus repeated bleeding; bled from aorta during the 3rd, 7th, 11th and 15th min. of anesthesia; blood volume restored | 15 min. | 60 | 0.005 pituitary<br>0.00125 pituitary | 106, 83, 164, 102<br>6, 65, 38, 45, 45, 54, 29 | 114<br>40 | 157.4±33. |
| | U.S.P. Standard ACTH | | | 1.0 mu.<br>0.5 | 97, 105, 88, 101, 52, 178<br>73, 77, 132, 30, 23, 26, 104, 25, 39, 93, 83, 77 | 104<br>65 | |
| 20 | Intact: continuous ether anesthesia plus repeated bleeding; bled from aorta during 3rd, 7th, 11th, and 15th min. of anesthesia; blood volume not restored | 15 min. | 37 | 0.01 pituitary<br>0.005 pituitary<br>0.0025 pituitary | 84, 63, 84, 154, 88, 59<br>25, 65, 67, 74, 10, 57<br>27, 11, 10, −18, −24 | 89<br>50<br>1 | 75.4±15.8 |

APPENDIX III—*Continued*

| Assay No. | Experiment | Duration of stress | Number of donor rats | Dose per 100 g. assay rat | Ascorbic acid depletion mg./100 g. fresh adrenal individual responses | Avg. | Content mu.* per pituitary M.± S.E.M. |
|---|---|---|---|---|---|---|---|
| | U.S.P. Standard ACTH | | | 1.0 mu. | 147, 79, 183, 162, 164 | 145 | |
| | | | | 0.5 | 113, 85, 118, 80, 82 | 96 | |
| | | | | 0.25 | 67, 74, 39, 80, 98, 117 | 79 | |
| 21 | Intact: decapitated without stress | None | 9 | 0.01 pituitary | 98, 111, 111, 108, 175, 141 | 124 | 64.4± 13.2 |
| | | | | 0.005 pituitary | 47, 58, 132, 78, 89 | 78 | |
| | | | | 0.0025 pituitary | 33, 35, 35, 74, 86 | 53 | |
| | U.S.P. Standard ACTH | | | 1.0 mu. | 180, 190, 141, 192, 138 | 168 | |
| | | | | 0.5 | 103, 125, 32, 89 | 88 | |
| | | | | 0.25 | 47, 28, 52, 37, 26 | 38 | |
| 22 | Intact: decapitated without stress | None | 6 | 0.01 pituitary | 138, 133, 62, 157, 157 | 129 | 80.1± 11 |
| | | | | 0.005 pituitary | 89, 74, 48, 55, 129 | 79 | |
| | | | | 0.0025 pituitary | 24, 16, 45, 68 | 38 | |
| | U.S.P. Standard ACTH | | | 1.0 mu. | 94, 145, 94, 100, 139 | 115 | |
| | | | | 0.5 | 77, 63, 67, 132, 156 | 88 | |
| | | | | 0.25 | 96, 53, 47, 110, 73, 69 | 75 | |
| 23 | Adrenx *2 wk:* decapitated without stress | None | 6 | 0.005 pituitary | 115, 147, 209, 143, 98, 66 | 142 | 161 ±79 |
| | | | | 0.0025 pituitary | 115, 109, 103, 129, 113, 43, 15, 23 | 80 | |
| | | | | 0.00125 pituitary | 47, 22, −17, 27, 18 | 19 | |
| | U.S.P. Standard ACTH | | | 1.0 mu. | 130, 101, 122, 65, 98 | 103 | |
| | | | | 0.5 | 58, 56, 75, 61, 121, 85 | 76 | |
| | | | | 0.25 | 17, 76, 68, 69, 13, −10 | 39 | |
| 24 | Adrenx *2 wk:* continuous ether anesthesia plus rapid exsanguination from aorta | 15 min. | 6 | 0.005 pituitary | 104, 100, 113, 70, 97 | 97 | 171.8+30.4 |
| | | | | 0.0025 pituitary | 52, 67, 41, 94, 68, 95 | 70 | |
| | | | | 0.00125 pituitary | 16, 19, 29, 33, 51 | 30 | |

* One millinnit (mu.) equals one-thousandth of a U.S.P. unit of ACTH (La-1-A).
† Estimate of potency based on single-dose assays.

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# COMPARISON OF THE DIABETOGENIC ACTION OF CORTISONE AND GROWTH HORMONE IN DIFFERENT SPECIES

WILLIAM A. ABELOVE,[1] AND KARL E. PASCHKIS,
*Division of Endocrine and Cancer Research, Jefferson Medical College, Philadelphia, Pennsylvania*

## INTRODUCTION

IN RECENT years a considerable amount of information has accumulated regarding the role of adrenocrotical hormones and of growth hormone[2] in carbohydrate metabolism and regarding the diabetogenic action of these agents. The diabetic response differs in different species. The present study was undertaken because it was hoped that a further study and more detailed evaluation of such species differences of response might contribute to an understanding of the mechanism of the diabetogenic hormone action.

## MATERIALS AND METHODS

The animals used in the experiments included 2 female mongrel dogs (weights 13.0 and 15.7 kg.), 4 female cats (weights from 1.75 to 3.3 kg.), 9 male rabbits (weights from 2.5 to 3.5 kg.), and 31 male guinea pigs (weights from 250 to 350 gm.).

All animals were kept in metabolism cages in an air-conditioned room (temperature 70–75° F.). Urine specimens were collected for consecutive twenty four hour periods and analyzed for glucose by "clinitest" tablets and by the method of Benedict. Blood specimens were obtained from the leg veins of the dogs, ear veins of the cats and rabbits, and hearts of the guinea pigs. Glucose determinations were done by a micro method using the anthrone reagent (Seifter *et al.*, 1950). The dogs only were fasted for 16 hours prior to drawing blood for blood sugar determinations.

One dog was fed a Kennel ration of meat and milk; the other was maintained on a high carbohydrate diet[3] with small amounts of meat to stimulate the appetite. Toward

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[1] Fellow of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, U. S. Public Health Service.

[2] The action of highly purified growth hormone preparations in carbohydrate metabolism will be discussed in this paper in terms of an action of growth hormone. If the claim that a carbohydrate-active (diabetogenic) factor can be separated in vitro from the growth promoting moiety (Raben *et al.*, 1952) will be confirmed, this would not detract from the discussion and conclusions presented in this paper.

[3] High carbohydrate diet: Casein 4.73, Sucrose 18.60, Dextrose 38.20, Dextrin 18.50, Lard 15.10, Wesson salt mixture 1.95, Agar 2.92.

Vitamin supplements (mg./day): Thiamin HCl (0.36), Riboflavin (0.3), Nicotinic acid (3.0), Ca Pantothenate (2.4), Pyridoxine (0.18), Choline (180) 2-methylnaphthoquinone (0.00012), Alpha tocopherol (6.0), Biotin (0.12), Folic acid (0.96), Vitamin A and D.

the end of the experiment 10% glucose solution was given as drinking solution instead of water. The cats received a ground meat and "Trim meal" diet with a daily supplement of four ounces of milk. The rabbits and guinea pigs were fed "Purina rabbit chow;" the guinea pigs received a daily subcutaneous injection of 10 mg. of ascorbic acid. Sterile solutions of 10% and 20% glucose were prepared and kept refrigerated for intraperitoneal administration to the guinea pigs. The solution was warmed to body temperature and injected intraperitoneally, twice daily (9 A.M., and 5 P.M.,) in doses up to 3 gm. per day. All animals received water *ad libitum*.

Hormone injections were given subcutaneously, twice daily (9 A.M. and 5 P.M.). Cortisone acetate suspension was supplied by Merck and Company. Growth hormone preparations were obtained from the Armour Laboratories (lot no. 285–183) and from The Wilson Laboratories (lot no. 85570).

Several guinea pigs were subjected to subtotal pancreatectomy under ether anesthesia, at which time 70–80% of the pancreas was removed.

Specimens from the pancreas and liver of dogs, guinea pigs, and cats, obtained either by biopsy, or at autopsy, were fixed in Bouins's solution and in absolute alcohol, and stained with hematoxylin and eosin, Best's carmine and Gomori's stain.

## RESULTS

### *Cats*

Results are summarized in Table 1. Cortisone in doses up to 200 mg./day (80 mg./kg) had only slight diabetogenic action in cats. During the thirty-one day period of the experiment one animal received a total of 3000 mg. and another 2650 mg. of cortisone acetate. A slight elevation in the blood sugar, polyuria, and occasional traces of glycosuria resulted (Table 1 and Fig. 1).

Growth hormone (Armour lot 285–183) was given to two cats; the small doses employed, 2.5 mg, and 5 mg. per day respectively, caused mild hyperglycemia, with occasional traces of glucose in the urine. There was oliguria, possibly due to contamination of the growth hormone preparation with posterior pituitary hormones (Table 1 and Fig. 2).

This same dose of growth hormone was administered to the two cats treated with cortisone. In this experiment, 100 mg./day of cortisone was injected; simultaneously growth hormone was given at two dose levels, 2.5 and 5 mg./day. This resulted in marked hyperglycemia, glycosuria, and weight loss (Table 1 and Fig. 1). It should be noted that the glycosuria was greater in the cat receiving the larger dose of growth hormone; the severe diabetic manifestations waned when growth hormone treatment was discountinued while cortisone administration was continued.

In the two cats treated with 2.5 and 5.0 mg./day of growth hormone, daily injections of 100 mg./day of cortisone were added. This was followed by a more marked rise of blood sugar, but without glycosuria (Table 1., Fig. 2).

TABLE 1. EFFECT OF ADMINISTRATION OF CORTISONE ACETATE AND OF GROWTH HORMONE IN CATS

| Cat No. | Cortisone ac. | | Growth h.* | | Blood sugar mg. % | Urine sugar† gm/24 hrs. | Urine vol.‡ ml./24 hrs. | Weight |
|---|---|---|---|---|---|---|---|---|
| | Dose/ day | Duration (days) | Dose/ days | Duration (days) | | | | |
| | 0 | | 0 | | 89 | 0 | 81 | 2880 |
| | 50 | 12 | 0 | | | 0 | 119 | 3010 |
| 1 | 100 | 14 | 0 | | 144 | trace | 119 | 3550 |
| | 200 | 5 | 0 | | 174 | 0 | 154 | 3660 |
| | 100 | 5 | 0 | | 100 | 0 | 123 | 3650 |
| | 100 | 12 | 5 | 12 | 285 | 12.5 | 127 | 3590 |
| | 0 | | 0 | | 100 | 0 | 71 | 1740 |
| | 50 | 19 | 0 | | 139 | 0.72 | 144 | 2260 |
| 2 | 100 | 7 | 0 | | 148 | 0 | 123 | 2520 |
| | 200 | 5 | 0 | | 132 | 0.36 | 149 | 2530 |
| | 100 | 12 | 0 | | 188 | 0.29 | 128 | 2610 |
| | 100 | 9 | 2.5 | 9 | 300 | 2.0 | 68 | 2240 |
| | 0 | | 0 | | 100 | 0 | 52 | 2490 |
| 3 | 0 | | 5 | 8 | 151 | 0.28 | 37 | 2610 |
| | 100 | 7 | 5 | 7 | 188 | 0 | 52 | 1430 |
| | | | | | 143 | | | |
| | 0 | | 0 | | 89 | 0 | 62 | 3310 |
| 4 | 0 | | 2.5 | 7 | 172 | trace | 49 | 3390 |
| | 100 | 7 | 2.5 | 7 | 226 | trace | 38 | 3190 |
| | | | | | 211 | | | |

* Armour lot 285–183.
† Maximum during respective periods.
‡ Mean during respective periods.

*Dogs*

Results are summarized in Table 2. As can be seen, in Dog 1, fed meat and milk, the daily dose of cortisone acetate was gradually inceased from 25 mg/day to 200 mg./day (12.5 mg./kg.). This animal was treated for 35 days. During the first 5 days, on 25 mg./day, no glycosuria occurred;

TABLE 2. EFFECT OF CORTISONE ACETATE ADMINISTRATION IN FEMALE DOGS

| Dog No. | Diet | Control period | | | Cortisone ac. s.c. mg./day | Treatment period | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Wt. (Kg.) | Fasting blood sugar, mg.% | Urine vol., max./24 hrs. | | Wt. (Kg.) | Fasting blood sugar, mg. % | Urine vol., max./24 hrs. | Glycosuria gm./24 hours |
| 1 | Stock (meat, milk) | 15.7 | 89 | 650 | 25– 5 days<br>50–17 days<br>100– 6 days<br>200– 7 days | 18.5 | 195 | 2040 | 1–2 |
| | | | | | 2975 in 35 days | | | | |
| 2 | High carbohydrate* | 11.0 | 88 | | 50– 6 days<br>100– 9 days<br>125–11 days<br>200–18 days | 17.5 | 100 | | 0 |
| | | | | | 6175 in 44 days | | | | |

* For composition see footnote 3.

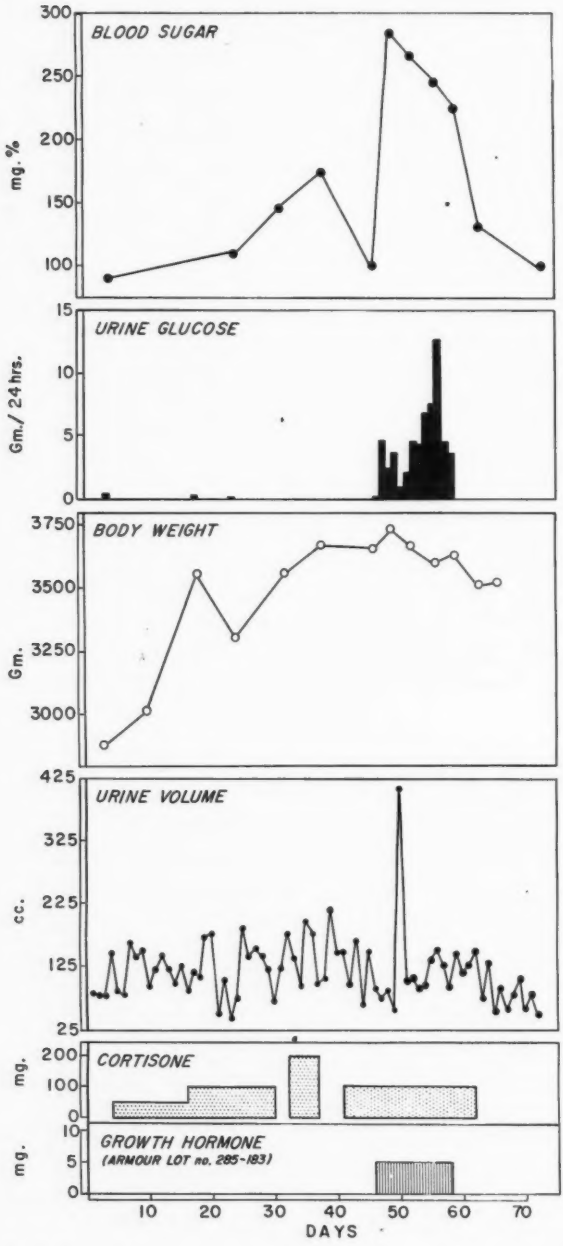


FIG. 1. Action of cortisone ac. and of growth hormone plus cortisone ac. in the cat (cat #2).

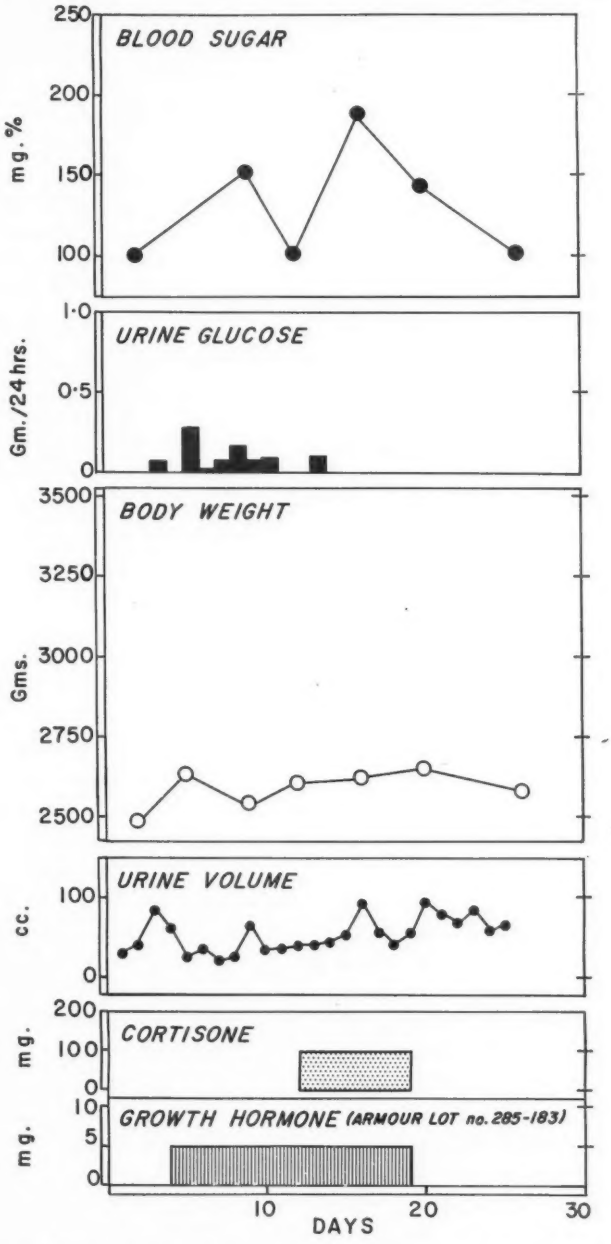


FIG. 2. Action of small dose of growth hormone, and of cortisone plus growth hormone in the cat (cat #4).

when the dose was raised to 50 mg./day, glucose, up to 1.0 mg./24 hours, was excreted and the blood sugar rose to 195 mg/100 ml. Further increases of the daily dose of cortisone to 100 mg. and 200 mg. caused no increase of glycosuria, but the urine volume rose to 2000 ml./24 hours. Cortisone administration was discontinued after 35 days; glycosuria up to 1.0 gm./24 hours persisted for the next two weeks. Nineteen days after treatment was discontinued, the blood sugar had returned to a value of 97 mg./100 ml. The weight of this dog remained constant (15.7 kg.) while receiving up to 100 mg./day of cortisone for 28 days; when the dose was increased to 200 mg./day the weight rose abruptly to 18.6 kg., but decreased promptly within 4 days after treatment was discontinued. Two biopsies of the pancreas were obtained, one on the 15th day of treatment, when the blood sugar was 195 mg./100 ml; a second on the 28th day of treatment. No significant changes of the islets were detected.

Dog 2, fed a high carbohydrate diet, was treated with cortisone in doses from 50 mg. to 200 mg./day; in 44 days a total of 6175 mg. were administered. During the last 7 days 10% glucose was given as drinking solution in lieu of tap water. During the entire period there was no glycosuria and the blood sugar never rose above 100 mg./100 ml. The weight rose from 11 kg. to 17½ kg.

*Guinea pigs*

Experiments are summarized in Table 3; Figures 3, 4 and 5 give data on representative experiments.

Thirteen guinea pigs received cortisone injections in daily doses of 2.5 to 25 mg. The smallest dose of 2.5 mg./day was administered to two animals for 14 and 11 days respectively; the blood sugar rose to 189 and 200 mg./100 ml. but there were only occasional traces of glycosuria.

Five animals received 5 mg./day of cortisone for periods between 10 and 30 days; all exhibited moderate hyperglycemia (maximum blood sugar values 125, 154, 174, 177, 236 mg. %), and 2 of the 5 showed moderate glycosuria with excretion between 0.5 and 2.0 gm. of glucose per day. Animals receiving the larger doses of 10, 15, 20 and 25 mg./day all had hyperglycemia (177–285 mg. %), glycosuria (0.23–7.2 mg. %), and polyuria (90–260 ml./24 hr.) (Fig. 3). Food and water intake were increased, but exact records were not kept. In five incidences, the hyperglycemia and glycosuria waned under continued administration of the same dose of cortisone, but waxed upon increase of the dose. All animals gained weight, even while receiving the large doses of cortisone, but the weight gain was less than that of untreated controls. Hyperglycemia persisted up to two weeks and glycosuria up to one week after treatment was discontinued; in no case was a permanent, or "metasteroid" diabetes observed. In 5 animals the blood sugar

TABLE 3. EFFECT OF CORTISONE ACETATE AND OF GROWTH HORMONE IN GUINEA PIGS

| No. of animals | Control period | | | Treatment period I | | | | | | Treatment period II | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Bl. sugar, mg. % | Urine vol., ml./day | Wt., gm. | Compound, dose/day | Duration, days | Bl. sugar, mg. % | Urine sugar, gm./day | Urine vol., ml./day | Wt., gm. | Compound, dose/day | Duration, days | Bl. sugar, mg. % | Urine sugar, gm./day | Urine vol., ml./day | Wt. gm. |
| 13 | 59–132 | 12–63 | 292–499 | cortisone ac. | 10–87 | 176–286 | 0.05–7.2 | 45–260 | 343–618 | | | | | | |
| | M=166 | M=32 | M=363.6 | 5–20 mg. | M=38 | M=218 | M=2.64 | M=138 | M=487.5 | | | | | | |
| 2 | 100 | 55–70 | 348–387 | pancreatect. cortisone ac. | 43–63 | 297–341 | 1.4 –4.8 | 139–280 | 551–625 | | | | | | |
| | | M=62.5 | M=367.5 | 5–15 mg. | M=53 | M=310 | M=3.1 | M=209 | M=588 | | | | | | |
| 6 | 71–121 | 15–70 | 324–514 | growth h.* | 10–21 | 125–261 | 0–0.07 | 38–70 | 376–536 | | | | | | |
| | M=99 | M=29 | M=429 | 2.5–10 mg. | M=14.5 | M=184 | M=0.03 | M=50 | M=471 | | | | | | |
| 2 | 100 | 40–53 | 315–349 | glucose i.p. 1–3 gm. | 14–47 | 254–266 | 0.16–0.84 | 47–128 | 375–505 | gluc. i.p. 1–3 gm. | 7 | 138–212 | 0.46–0.48 | 64–100 | 382–495 |
| | | M=47.5 | M=332 | | | M=260 | M=0.5 | M=87.5 | M=440 | growth h.† 25–50 mg. | | M=175 | M=0.47 | M=82 | M=438.5 |
| 3 | 115–121 | 17–40 | 354–412 | cortisone ac. 5 mg. | 10 | 177–280 | 1–3.5 | 26–66 | 400–469 | | | | | | |
| | M=119 | M=26 | M=379 | growth h.* 1.25–5 | | M=226 | M=2.5 | M=47 | M=428 | | | | | | |
| 1 | 121 | 35 | 346 | cortisone ac. 2.5 mg. | 14 | 189 | 0.06 | 90 | 417 | cortisone ac. 2.5 mg. growth h.* 5 mg. | 7 | 163 | 0.07 | 100 | 436 |
| 1 | 110 | 15 | 389 | growth h.* 2.5 mg. | 14 | 249 | 0 | 65 | 472 | growth h.* 2.5 mg. cortisone ac. 2.5 mg. | 7 | 143 | 0.02 | 60 | 470 |
| 2 | 110 | 25–40 | 350–376 | cortisone ac. 2.5 mg. | 14 | 160–162 | 0.04–0.5 | 45–68 | 405–463 | cortisone ac. 2.5 mg. | 4 | 163–189 | 0 | 40–55 | 439–449 |
| | | M=32.5 | M=363 | growth h.* 2.5, 5.0 mg. | | M=161 | M=0.27 | M=56.5 | M=420.5 | growth h.* 5.0, 10 mg. | | M=176 | | M=47.5 | M=444 |

* Wilson, Lot No. 85570.
† Armour, Lot No. 285–183.
Note: Values recorded for Blood sugar, Urine sugar, Urine volume and weight are the highest values found during the respective period.

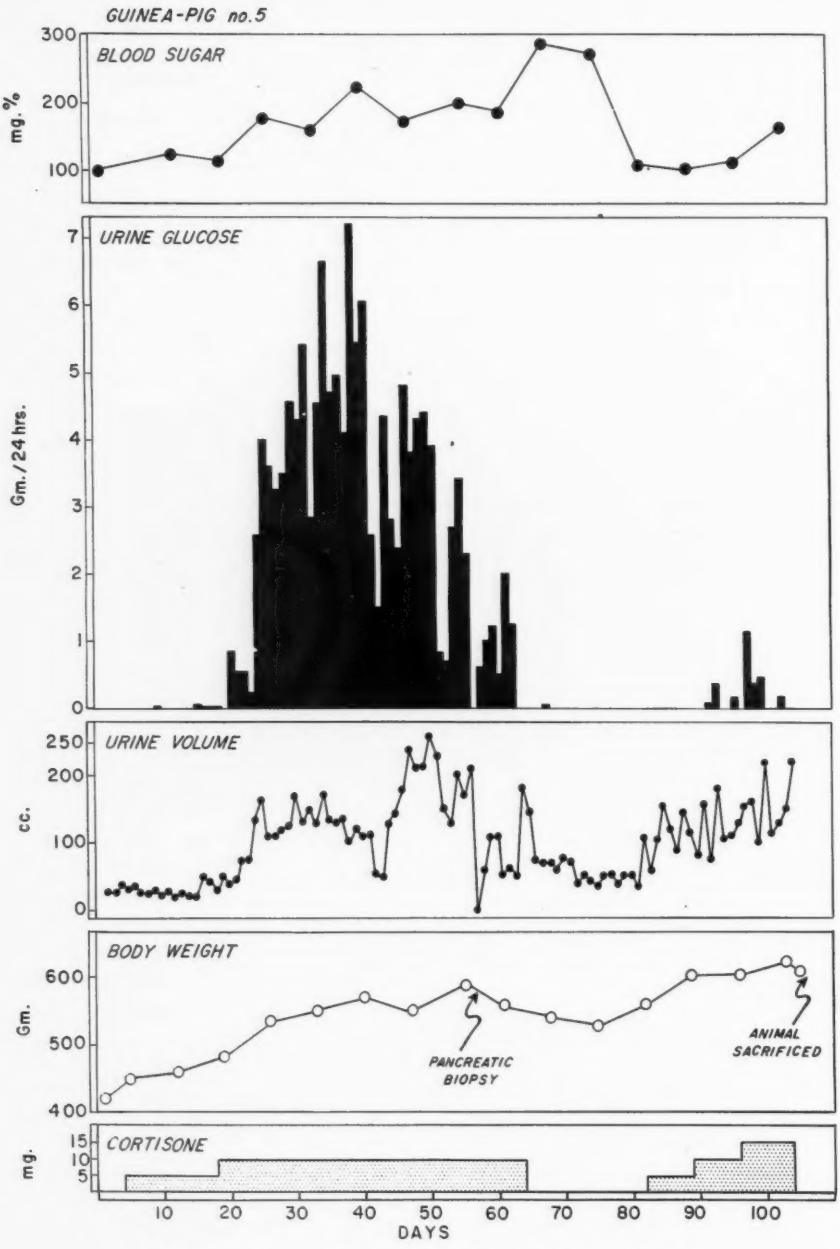


FIG. 3. Cortisone ac.-ind ced diabetes in the guinea pig.

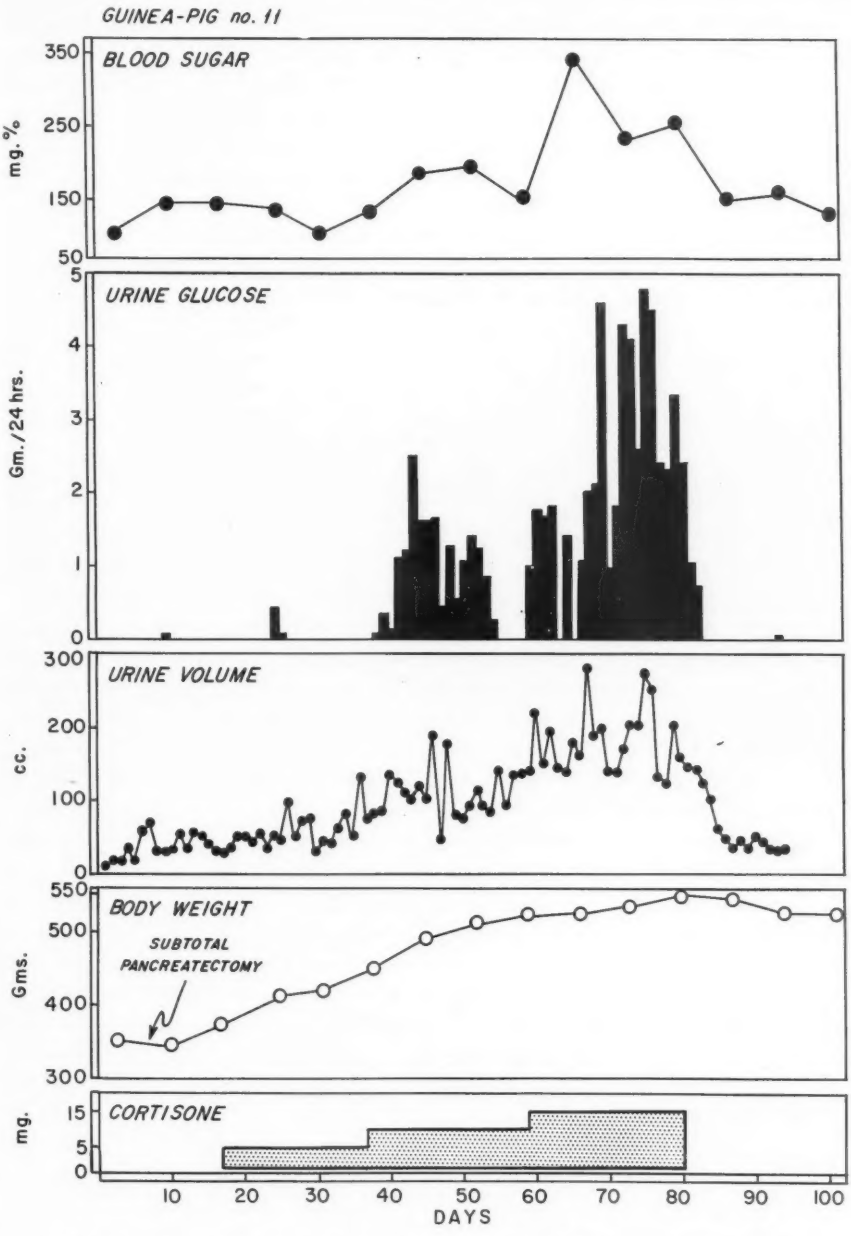


FIG. 4. Cortisone ac.-induced diabetes in the partially depancreatized guinea pig.

decreased to or approached normal values, and glycosuria disappeared while on a previously "diabetogenic" dose; an increase in the dose of cortisone was again effective in reinstating the diabetes. There was some variation of the dose, and length of treatment necessary to induce diabetes. Tissue for histological study was obtained from seven guinea pigs, either at autopsy, or by biopsy. Treatment with cortisone caused an enormous increase in size and number of Langerhans islets.

Two guinea pigs were subjected to partial pancreatectomy. It was estimated that about 75% of the gland was removed. This caused neither hyperglycemia nor glycosuria. Subsequently both animals were treated with cortisone in daily doses of from 5 to 15 mg. The diabetic response did not differ from that observed with the same dosage in intact animals. (Fig. 4).

Six guinea pigs received growth hormone in doses ranging from 2.5 to 10 mg./day, for periods from 10–21 days. This resulted in transient hyperglycemia, up to 261 mg./100 ml. In three instances the hyperglycemia was not maintained under continued treatment. Traces only of glucose appeared in the urine. There was no increase in urine volume. All animals gained weight, but to a lesser degree than untreated controls. Two animals received daily intraperitoneal injections of glucose, in amounts from 1 to 3 gm. per day for 21 to 54 days respectively; during the last 7 days, growth hormone was administered subcutaneously (50 and 25 mg./day respectively) concomitantly with the intraperitoneal glucose injections. Growth hormone did not cause an increase of the hyperglycemia induced by the intraperitoneal glucose administration, nor did it increase the slight glycosuria (Fig. 5). During the glucose administration the animals gained 4 and 3 gm. per day. During the week of combined glucose and growth hormone treatment one animal gained 1 gm. per day, the other lost 1.4 gm. per day.

Seven guinea pigs received combined treatment with cortisone and growth hormone. Three of these animals received 5 mg. of cortisone per day plus 1.25, 2.5 and 5.0 mg./day of growth hormone respectively, for 10 days. Two others received 2.5 mg. of cortisone per day and growth hormone in doses of 2.5 to 10 mg. for 18 days. One animal was treated with 2.5 mg. of cortisone for 21 days, and 5 mg. of growth hormone were added during the last 7 days; the seventh animal received 2.5 mg. of growth hormone per day for 21 days, and 2.5 mg. of cortisone per day were added for the last 7 days. With none of these combinations was there any clear-cut evidence of "synergism."

*Rabbits*

Experiments are summarized in Table 4.

Cortisone in doses as small as 2.5 mg. per day (approximately 1 mg./kg.) induced hyperglycemia and glycosuria in three of four male rabbits on

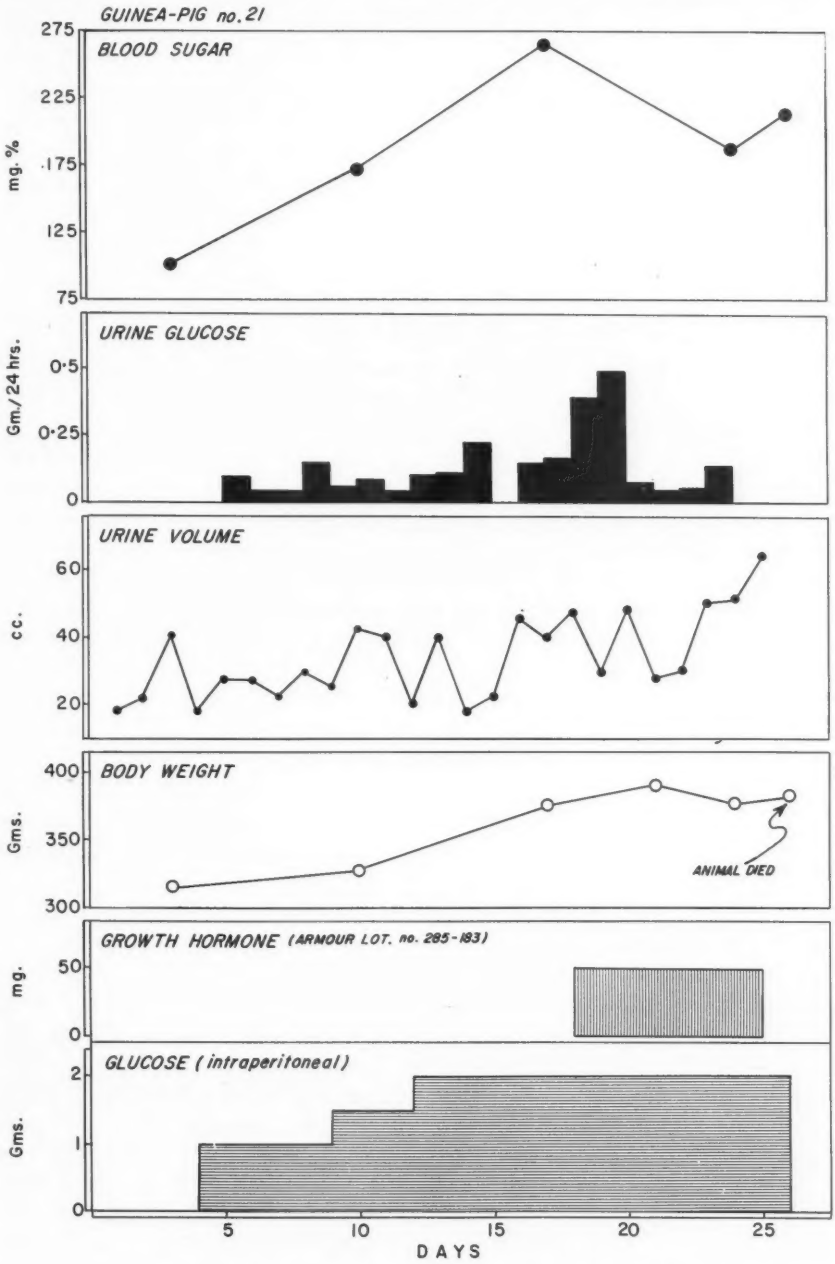


FIG. 5. Effect of growth hormone in a guinea pig receiving glucose intraperitoneally.

TABLE 4. ACTION OF CORTISONE ACETATE AND OF GROWTH HORMONE IN THE RABBIT

| Control period | | | | Treatment period I | | | | | | | Treatment period II | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Rabbit No. | Bl. sugar, mg. % | Urine vol., cc./day | Wt., kg. | Compound | Dose/day, mg. | Duration | Bl. sugar, mg. % | Urine sugar, gm./day | Urine vol., ml./day | Wt., kg. | Compound | Dose/day, mg. | Duration | Bl. sugar, mg. % | Urine sugar, gm./day | Urine vol., ml./day | Wt., kg. |
| 1 | 111 | 66 | 2.7 | cortisone ac. | 2.5 | 27 | 273 | 1.93 | 125 | 2.78 | | | | | | | |
| | | | | | 5.0 | 7 | 185 | 0.98 | 150 | 2.77 | | | | | | | |
| | | | | | 10.0 | 6 | 213 | 0.81 | 150 | 2.75 | | | | | | | |
| 2 | 100 | 125 | 3.19 | cortisone ac. | 2.5 | 8 | 154 | 0.38 | 150 | 3.42 | | | | | | | |
| | | | | | 5.0 | 4 | 155 | 0.31 | 125 | | | | | | | | |
| | | | | | 15.0 | 4 | 166 | 0.38 | 125 | 3.22 | | | | | | | |
| 3 | 90 | 135 | 2.61 | cortisone ac. | 2.5 | 11 | 294 | 3.6 | 220 | 2.74 | | | | | | | |
| 4 | 100 | 125 | 2.77 | cortisone ac. | 5.0 | 8 | 132 | 0.25 | 150 | 2.67 | cortisone ac. | 5.0 | 7 | 171 | 0.33 | 145 | 2.37 |
| | | | | | | | | | | | growth* | 5.0 | | | | | |
| 5 | 100 | 140 | 3.09 | growth* | 2.5 | 12 | 177 | 0.18 | 200 | 3.27 | growth* | a 2.5 | 15 | 620 | 3.03 | 200 | 3.17 |
| | | | | | | | | | | | | b 5.0 | 7 | 155 | 0 | 320 | 313 |
| | | | | | | | | | | | cortisone ac. | 2.5 | 22 | | | | |
| 6 | 100 | 145 | 2.97 | growth* | 5.0 | 27 | 154 | 0.30 | 275 | 3.55 | growth* | 5.0 | 9 | 144 | 1.08 | 280 | 3.43 |
| | | | | | | | | | | | cortisone ac. | 2.5 | | | | | |

* Wilson lot No. 85570.

stock diet. Resistance to the steroid developed in all cases within 9 to 28 days, as evidenced by diminution, under continued treatment, of glycosuria and return of blood sugar levels to normal. By increasing the dosage in one animal, the resistance was overcome. The total urine volume increased markedly during cortisone administration in 2 of 4 animals. The weight remained constant during the experimental period.

Growth hormone in doses of 1–2 mg./kg/day produced hyperglycemia, and traces of glycosuria in two rabbits on stock diet. Under continued hormone administration, the elevated blood sugar returned to normal within two weeks in one animal, whereas in the other it decreased somewhat but still remained elevated (160 mg./100 ml.). There was no significant glycosuria, but some increase in urine volume. The animals showed slight weight gain during the course of the experiment.

Combined treatment with both hormones was carried out in 3 rabbits. One of these was pretreated with 5 mg./day of cortisone for 8 days; during the next 7 days cortisone administration was continued, and 5 mg./day of growth hormone were given simultaneously. The latter failed to intensify the mild diabetes induced by cortisone. The two other animals were pretreated with growth hormone in daily doses of 2.5 mg. (12 days) and 5 mg. (27 days) respectively. Under continued treatment with growth hormone, cortisone administration was carried out for 22 and 9 days respectively. In the animal receiving 5 mg. of growth hormone, additional cortisone treatment was ineffective. In the other rabbit, receiving 2.5 mg. of growth hormone, addition of cortisone caused increased hyperglycemia and marked glycosuria. These effects disappeared on the 11th day of the combined treatment; on the 15th day the daily dose of growth hormone was increased to 5 mg. whereas the dose of cortisone was kept at the level of 2.5 mg. The increased dose of growth hormone caused no further changes.

## DISCUSSION

The differences in response to the two diabetogenic hormones in different species are apparent from the findings presented in this paper, which in part confirm, and extend data reported by others. Our findings of considerable diabetic response to cortisone administration in guinea pigs confirm those of Hausberger and Ramsay (1953). Our finding of islet hyperplasia also confirms their data. Guinea pigs continue to gain weight under treatment with cortisone, but the weight gain is smaller than that of untreated controls; it is of interest that in Hausberger's experiments on larger animals which showed constant weight during the control period, weight gain was observed during cortisone treatment. It would appear that cortisone causes relative weight loss (diminished gain) in growing animals, and weight gain in plateaued, full grown guinea pigs. Partial pancreatectomy

(about 75%) failed to increase the severity of cortisone-induced diabetes in guinea pigs; the possible significance of this finding will be discussed later. In all instances glycosuria disappeared and blood sugar levels returned to normal after discontinuing cortisone administration; there was no instance of "metasteroid" diabetes.

Rabbits also developed hyperglycemia and glycosuria under treatment with cortisone; the effective doses were smaller than those employed by Kobernik and Moore (1950). In our experiment the animals developed intense polyuria; their weight remained constant. There appeared to be no marked developed intense polyuria; their weight remained constant. There appeared to be no marked islet cell hyperplasia as described above in guinea pigs; nor did we observe hydropic degeneration of the islet cells as described by Kobernik and Moore. However, histologic studies were not made in all our animals, and this aspect needs further study. As in the guinea pigs, there was no instance of metasteroid-diabetes.

One of our two dogs developed mild steroid diabetes during cortisone treatment, whereas a second dog remained normoglycemic and aglycosuric with cortisone doses up to 200 mg. per day. Interestingly, the resistant dog was fed a high carbohydrate diet, whereas the responsive animal received kennel ration of meat and milk. No final conclusions can be drawn from comparison of only two animals, but it would appear that the independance of diabetogenic action from the dietary carbohydrate levels in these dogs differs from findings reported in the rat by Ingle who found cortisone (1941) and ACTH (1946) diabetogenic only in animals fed high carbohydrate diets. However, in later studies a diabetogenic effect of cortisone was observed also in rats fed average levels of carbohydrate ("medium carbohydrate diet") (Ingle *et al.*, 1952, 1953). Cortisone failed to induce diabetes in dogs in the experiments of Mushett *et al.* (1951) and Sirek and Best (1952). ACTH was also reported to be ineffective in this species by Overbeek (1950), but, in large doses, did induce mild diabetes, in intact dogs (Houssay and Anderson, 1949; Campbell, *et al.*, 1950; Fajans, *et al.*, 1951) and in partially depancreatized dogs (Houssay and Anderson, 1949). From the data available it would seem that perhaps a diabetogenic response to ACTH occurs somewhat more readily than to cortisone. This would be in agreement with the findings of DeBodo (1953) who stated that hypophysectomized dogs are quite sensitive to the diabetogenic action of ACTH, but not of cortisone or hydrocortisone.

Our cats treated with cortisone showed only mild hyperglycemia and occasional glycosuria. Others have found ACTH to have no diabetogenic effect in intact cats (Li, 1949–1950; Houssay and Anderson, 1949; Reid, 1951); partial pancreatectomy also failed to render the cat sensitive to ACTH (Houssay and Anderson, 1949). However, ACTH was strongly

diabetogenic in the depancreatized-hypophysectomized cat (Milman *et al.*, 1951).

In experiments reported in this paper growth hormone induced mild hyperglycemia and glycosuria in guinea pigs and rabbits; in the guinea pig growth hormone failed to intensify the hyperglycemia and mild glycosuria induced by intraperitoneal glucose administration. In two growth hormone treated guinea pigs the pancreatic islets were not hypertrophied. Ogilvie induced diabetes in rabbits with crude saline anterior pituitary extract. There was hypertrophy, and in one instance an increase of the number of, pancreatic islets (Ogilvie, 1944). The mild, though not completely absent, diabetogenic response to growth hormone in guinea pig and rabbit contrasts clearly with the marked diabetogenic effect of growth hormone in dogs (Campbell *et al.*, 1950, 1953; Young 1953) and cats (Cotes *et al.*, 1949).

In rats growth hormone is diabetogenic only after partial pancreatectomy (Marx *et al.*, 1953; Young, 1951).

This comparison of the response to the two hormones in different species leads to certain general comments. It would appear that all species investigated, respond to the administration of either hormone, and that the differences are quantitative rather than qualitative. The dog and cat which are "resistant" to the diabetogenic action of adrenal steroids, do show a mild response and are thus only relatively resistant. Equally the rodents (rabbit, guinea pig, rat) are not completely resistant to the diabetogenic action of growth hormone. Some form of "synergism" between action of the adrenal steroids and growth hormone can be demonstrated in several species; it was found for growth hormone and ACTH in the rat (Engel, 1952) and in the cat (Reid, 1951, 1953a, 1953b), and for growth hormone and cortisone in the cat (this paper). We did not see any indication of synergism of growth hormone and cortisone in the rabbit or guinea pig under the conditions of the experiments. The findings of a synergistic action of administered exogenous hormones in the intact animal do not answer the question whether synergism is obligatory for a diabetic effect, or whether there is an independent action of either. The latter, indeed, appears to be the case in the dog; ACTH has been found diabetogenic in the hypophysectomized dog (DeBodo, 1953) and growth hormone in the adrenalectomized animal (Lockett *et al.*, 1953). In the cat, on the other hand, growth hormone is ineffective in the adrenalectomized animal (Lockett *et al.*, 1953), but ACTH is effective in the hypophysectomized cat (Milman *et al.*, 1951). With regard to the synergistic action of cortisone and growth hormone in the intact cat cortisone appears to be the "conditioning" or sensitizing factor, inasmuch as in our experiments the severity of the diabetes increased with increasing doses of growth hormone during a constant dose level of cortisone. A similar observation was made by Engel

*et al.* (1952) in the rat receiving ACTH and growth hormone; on a constant dose level of ACTH the severity of the diabetes varied with varying doses of growth hormone. In the experiments on cats reported in this paper the synergistic effect was much more marked when sub-effective doses of growth hormone were injected into cortisone pretreated cats, than when cortisone was injected into growth hormone pretreated animals.

From available data it would appear that partial pancreatectomy increases the diabetic effect of administered growth hormone in the cat, dog (Houssay and Anderson, 1949) and rat (Marx *et al.*, 1943; Young *et al.*, 1951). Contrariwise, partial pancreatectomy does not seem to increase the severity of cortisone or ACTH induced diabetes either in relatively insensitive animals (dog, cat: Houssay and Anderson, 1949) or in sensitive animals (guinea pig: this report). This aspect may be worthy of further study in more species since present data would suggest a different mode of action of the two diabetogenic hormones.

The mechanism of action by which growth hormone induces diabetes in dogs and cats has been explained by an inability to secrete the extra insulin necessary to control the hyperglycemia induced by a peripheral action of growth hormone, chiefly on carbohydrate utilization. The insulin content of the growth hormone treated dog decreases early (Campbell *et al.*, 1953), and both in dogs and cats there are progressive degenerative changes in the islets (Ham and Haist, 1941). This explanation receives strong support from the work of Lukens and Dohan who could prevent the islet changes by preventing growth hormone induced hyperglycemia with insulin or phlorrizin (Lukens and Dohan, 1942), and could on the other hand duplicate the growth hormone induced islet changes by raising the blood sugar levels by means of glucose administration (Dohan and Lukens, 1948). The relative resistance of the rat to the diabetogenic action of growth hormone is explained by the assumption that the rats islet cells can supply the extra insulin in response to the growth hormone induced hyperglycemia; there is early increase of insulin content (Haist and Kinash, 1951) and hyperplasia of the islets (Marks and Young, 1940). These observations have been tentatively applied to the explanation of certain aspects of human diabetes by assuming that some individuals have dog-like and others rat-like islets (Paschkis, Rakoff and Cantarow, 1954). However, some of the observations reported in this paper indicate that other factors probably play an additional role. The guinea pig is quite resistant to the diabetogenic action of growth hormone, but the pancreas of growth hormone treated guinea pigs does not present islet cell hyperplasia as does that of the gorwth hormone treated rat. On the other hand the severely diabetic cortisone-treated guinea pig shows marked islet hyperplasia (Hausberger and Ramsay, 1953, this report). Inability to supply necessary increased

amounts of insulin may then be associated with early severe degeneration of islets (growth hormone treated dogs and cats) or with hyperplasia and vacuolar degeneration of islets (cortisone treated guinea pigs). Relative resistance to the diabetogenic action of growth hormone may be associated with islet cell hyperplasia (rat) or with a normal pancreas (guinea pig). Further studies will show whether these differences are absolute, or a matter of hormone dosage employed in the respective experiments.

## SUMMARY

1. Cortisone acetate in doses up to 200 mg. per day, exerted only a very slight diabetogenic action in cats. Small doses of growth hormone (2.5 or 5.0 mg per day) produced only a moderate hyperglycemia. When such small doses of growth hormone were given simultaneously with cortisone acetate, a marked hyperglycemic and glycosuria developed. This synergistic effect was more marked in animals pretreated with cortisone and then with the combined treatment, than in those pretreated with growth hormone prior to the combined treatment.

2. Cortisone acetate in doses up to 200 mg. induced mild steroid diabetes in one dog on kennel ration (meat and milk), but failed to induce diabetes in a second dog fed a high carbohydrate diet.

3. Guinea pigs injected with cortisone acetate develop severe diabetes which persists for several days after treatment is discontinued. The diabetic guinea pigs showed marked increase in size and number of Langerhans islets. The severity of cortisone-induced diabetes was not greater in partially depancreatized animals. No permanent, metasteroid diabetes was observed in intact or partially depancreatized guinea pigs. Growth hormone caused only mild and transient hyperglycemia. Hyperglycemia and glycosuria induced by repeated intraperitoneal injection of glucose was not intensified by administration of growth hormone. Combined treatment with cortisone acetate and growth hormone failed to show any evidence of a synergistic effect.

4. In the rabbit cortisone acetate induced marked diabetes, whereas growth hormone caused only mild hyperglycemia. There was no synergistic effect upon combined treatment with the two agents.

### *Acknowledgments*

We are indebted for the generous supply of hormones to Dr. F. Alpert of Merck and Company (Cortone Acetate), Dr. E. Hays and Dr. S. L. Steelman of the Armour Laboratories (Growth Hormone) and to Dr. C. E. Graham of the Wilson Laboratories (Growth Hormone).

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# CONTINUATION OF PREGNANCY IN RHESUS MONKEYS (MACACA MULATTA) FOLLOWING HYPOPHYSECTOMY[1]

PHILIP E. SMITH

*Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York*

THE effect of the ablation of the intrasellar components of the pituitary gland (pars distalis, pars intermedia, infundibular process) on gestation has not been reported in monkeys except for a brief note (Smith, 1946) and a paper on the mammary glands (Agate, 1952). The extended period of gestation, as compared to other laboratory mammals, permits a long period to elapse between hypohysectomy and parturition, thus allowing the disabilities resulting from a pituitary deficiency to fully develop. In the forms reported by other investigators the ablation of the hypophysis before approximately mid-term has invariably resulted in abortion. In some species hypophysectomy at mid-pregnancy or later does not interrupt gestation; in others abortion occurs. In those species in which pregnancy continues, the longest period between operation and parturition has been only 11–12 days, Pencharz and Long (1933), and Selye, Collip and Thomson (1933) in rats; Selye, Collip and Thomson (1933), and Gardner and Allen (1942) in mice; Pencharz and Lyons (1934) in guinea pigs. In some of these forms the birth mechanism is disturbed with fetal mortality. In dogs hypophysectomy causes abortion (Aschner, 1912). In cats, Allen and Wiles (1932) report delivery of living young whereas McPhail (1935a) concludes that abortion occurs. In neither dogs nor cats were the pregnancies dated. In ferrets the findings are also inconclusive but indicate that abortion results (McPhail, 1935b). In rabbits hypophysectomy invariably causes abortion (White, 1932).

In rhesus monkeys as will be described in this paper, nearly 5 months or five sixths of the gestation period may elapse between hypophysectomy and parturition with approximately one half of the operated animals having fully developed offspring.

## EXPERIMENTAL PROCEDURE

Sexually mature rhesus monkeys were used. Most of the animals had been in our colony for one to several years; replacements were made from our Puerto Rico Primate

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[1] Aided by a grant from the Rockefeller Foundation.

Colony and from a dealer. The monkeys were kept in individual cages and were fed an abundant diet consisting of unground wheat, dog biscuits, oranges, bananas, lettuce and 2 hard-boiled eggs each week.

Daily observations, except Sundays, were made for menstruation or the "placental sign." Females were placed singly with males usually for a 4-day period in mid-cycle and if conception occurred the pregnancy was dated from day 12, the first day of menstruation being taken as day 1.[2] Thus the date of conception given in the tables may err as much as ±2 or in some cases ±3 days. Breeding was done during the fall and winter months for as Hartman (1932) has shown the rhesus monkey in captivity has an ovulatory cycle during the warm months.

A parapharyngeal approach, modified from that employed on rats (Smith, 1930), was used in removing the hypophysis. Under nembutal anaesthesia, supplemented by ether through a tracheal cannula, a parasagittal incision through the skin and platysma was made lateral to the larynx. The prevertebral fascia was reached, largely by blunt dissection, and traced crownward to the occipitosphenoid synchondrosis which is an important landmark. A tubular cavity through the body of the sphenoid bone to the sella was then made with dental burrs. The pituitary capsule (dural lining of the sella) was incised, using care not to separate it laterally from the bone and the intrasellar components of the hypophysis were removed by using a curette and a cannula with negative pressure. There was but little bleeding and a clear view of the evacuated sella was obtained. Following the removal of the hypophysis, the walls of the sella were sponged with 3–5% chromic acid to destroy any remaining fragment of the hypophysis and to prevent a growth of the pars tuberalis into the sella. Such a growth on later microscopic examination leaves a feeling of uncertainty whether the tissue is pars tuberalis or dedifferentiated anterior lobe tissue. The operations were done asceptically.

Following hypophysectomy 20 cc. of 50% glucose was given i.v. and usually also a 20 cc. transfusion of whole blood from a donor monkey. The operated animals were active and eating within a few hours after the operation.

In spite of shielding the operated animals from drafts a number were lost from pneumonia. The susceptibility to pneumonia after hypophysectomy was first noted by Aschner (1912) in dogs. No animals which died during pregnancy from pneumonia are included in the tables.

At autopsy the pituitary capsule after fixation *in situ* was carefully removed, sectioned serially, stained with Masson's trichrome and examined. No fragment of the intrasellar component of the hypophysis was present in any animal here reported. In 6 monkeys a block of tissue, encompassing the region in which a pharyngeal hypophysis constantly occurs in man, was serially sectioned and examined without finding any hypophyseal tissue.

## RESULTS

Table 1 shows that 10 of the animals hypophysectomized during pregnancy continued to term. The duration of pregnancy after hypophysectomy varied, in general, with the stage of gestation at operation. The

[2] The time of ovulation, determined by recovery of ova, manual palpation of the ovaries through the rectal wall, and by limited periods of mating has a considerable spread. Hartman (1932, 1944) reports ovulation between days 9 and 18, the greatest frequency being on days 12–13. Van Wagenen (1943) in an extensive series of matings places the mode at days 11–12, about 1 day earlier than Hartman.

TABLE 1. HYPOPHYSECTOMIZED MONKEYS WHICH WENT TO TERM

| Animal | Days preg. at hypx. | Days between hypx. & partur. | Gestation period. Days. | Body wt., gm. | | | | Baby | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | At conception | At hypx. | Maximum | At partur. | Wt., gm. | Sitting height, mm. |
| A | 32 | 131 | 163 | 4,760 | 5,000 | 6,140 | 5,600 | 370 ♀[1] | 194 |
| B | 50 | 130 | 180 | 5,650 | 5,900 | 8,000 | 6,330 | 406 ♂[2] | 215 |
| C | 74 | 85 | 159 | 6,880 | 7,320 | 7,480 | 6,360 | 230 ♂[3] | 164 |
| D | 78 | 88 | 166 | 4,500 | 4,800 | 5,730 | 5,560 | 310 ♀[4] | 172 |
| E | 102 | 59 | 161 | 5,870 | 6,900 | 7,200 | 6,400 | 320 ♀[5] | |
| F | 106 | 71 | 177 | 5,350 | 5,850 | 6,000 | 5,830 | 543 ♂[6] | 200 |
| G | 141 | 35 | 176 | 8,450 | 10,650 | 10,250 | 10,250 | 450 ♂[7] | 190 |
| H | 142 | 32 | 174 | 5,400 | 6,400 | 6,550[11] | 6,270 | 448 ♂[8] | 190 |
| I | 152 | 20 | 172 | 5,600 | 6,560 | 6,570[11] | 6,300 | 359 ♂[9] | |
| J | 156 | 35 | 191 | 7,600 | 9,400 | 9,450[11] | 7,500 | 430 ♂[10] | 200 |
| | | | Av. 171.9 | | | | | Av. 390.2 | Av. 190.4 |

[1] Baby healthy. Placenta expelled but not eaten. Cord not cut.
[2] Breech presentation. Head locked in cervix. Decapitated.
[3] Difficult labor. Immediate caesarean section. Baby healthy.
[4] Breech presentation. Head locked in cervix. Attempted to assist mother but baby died.
[5] Normal delivery. Placenta retained in vagina. Cord not cut.
[6] Difficult labor. Caesarean section. Baby healthy.
[7] Normal birth. Baby healthy. Placenta eaten and cord cut.
[8] Still birth. Baby recently died. Placenta not eaten. Cord not cut.
[9] Normal delivery. Baby healthy. Placenta not eaten. Cord not cut.
[10] Caesarean section. Baby recently dead. Head presentation. Cervix not dilated.
[11] Prehypophysectomy weight.

longest period was 161 ±2 or 3 days (animal *A*) or nearly five sixths of the gestation period. The shortest period in which a living baby was born after hypophysectomy was 20 days. During the posthypophysectomy period the gain in weight and antepartum loss of weight were within the normal range. The sex color was a brilliant as in intact pregnant monkeys. It extended onto the face and those regions of the body and legs normally displaying enhanced coloration. A surprising phenomenon was the slow regression in color following parturition.

From the 10 monkeys in which pregnancy continued to term after hypophysectomy 6 living healthy babies were obtained (*A*, *C*, *E*, *F*, *G*, *I*, Table 1). In two of these (*C*, *F*) the mothers were obviously too fatigued to continue in labor and delivery was by caesarean section. In the other 4 cases the babies were delivered after normal labor. Of the 4 cases in which nonliving babies were secured, 2 were breech presentations (*B*, *D*). The fetal heads of these were locked in the cervices and forcible removal was necessary, 1 requiring decapitation of the fetus and caesarean section. The babies were not macerated and undoubtedly were living when labor began. Two stillbirths are included in Table 1. They are included in this table rather than in Table 2 inasmuch as more than a month elapsed between the removal of the hypophysis and delivery. As will be pointed out later, stillbirths are not uncommon in intact monkeys.

TABLE 2. HYPOPHYSECTOMIZED MONKEYS WHICH ABORTED

| Animal | Days preg. at hypx. | Abortion, days after hypx. | Body weight, gm. | | |
|---|---|---|---|---|---|
| | | | At concept. | At hypx. | Just prior to abortion |
| K | 27 | ?[1] | 5,650 | 5,400 | |
| L | 29 | 58[2] | 5,300 | 5,300 | 5,830 |
| M | 32 | 51[3] | 5,700 | 5,600 | 5,940 |
| N | 33 | 23[4] | 4,900 | 4,900 | 5,100 |
| O | 39 | 44[5] | 5,500 | 5,550 | |
| P | 56 | 39[6] | 5,650 | 5,710 | 5,910 |
| Q | 66 | 7[7] | 5,350 | 5,550 | 5,600 |
| R | 101 | 4[8] | 4,500 | 5,100 | 5,100 |
| S | 142 | 10[9] | 4,600 | 5,200 | 5,200 |
| T | 156 | 4[9] | 7,500 | 8,220 | 8,200 |

[1] Pregnancy is probable but not certain from palpating the uterus.
[2] The fetus was not macerated and may have been living when aborted. Sitting height 100 mm. Placenta not eaten.
[3] The placenta and fetus were not macerated. Fetus weight 58 gm. Sitting height 110 mm.
[4] The placenta but not the fetus was found in the cage.
[5] A laparotomy 9 days before hypophysectomy revealed an enlarged uterus. No abortus found. At autopsy 111 days after hypophysectomy the adrenals were profoundly atrophied. The time of abortion is estimated from palpation of the uterus and color of the skin.
[6] Abortus not found but there was much blood in the cage.
[7] Placenta but no fetus found in the cage.
[8] This animal perhaps should not be included in the table for it did not actually abort. It seemed in excellent condition on the third day after hypophysectomy but was found dead the next morning. Its lungs and abdomen were clear and there was no inflammation of the meninges. Fetus 178 gm. Sitting height 135 mm.
[9] This could be classified as stillbirth. The fetus appeared to be fullterm and was not macerated.

In most of the cases in which living babies were delivered unassisted, the mothers were in a state of near collapse after parturition and 1 died 18 hours later. In no case was there excesssive hemorrhage as judged by observation during labor or by the condition of the cage when parturition occurred at night.

The postpartum behavior of the hypophysectomized mothers was abnormal in that they did not consume the afterbirth, except in 1 case. They would at times lick the placenta, and they vigorously resisted its removal from the cage. The umbilical cord was not cut even though a baby would be clinging to the hair of the mother's chest and thus would drag the placenta over the floor of the cage as the mother moved about. Normal mothers characteristically eat the afterbirth (Hartman, 1932). In 1 case the placenta was still in the vagina the morning after parturition. In other respects the mothers displayed normal maternal behavior. They zealously guarded their babies and fought against having them removed. In returning a baby to the mother after its temporary removal, care had to be used or she would reach through the cage bars and injure the infant by attempting to pull it into the cage. In monkeys the hypophyseal secretions appear to be not involved in the protective behavior towards the young.

Milk could be expressed from the nipples following parturition, in some

cases a stream several inches in length being secured. Lactation, however, was only temporary. The infants would suckle without success and if it was desired to keep them alive, bottle feeding was resorted to.

The resistance and health of the pregnant hypophysectomized monkeys seemed little if any better than in the nonpregnant ones after a corresponding period of apituitarism. In some cases their appetite was equal to that of an intact animal, whereas in others feeding by stomach tube or occasional intravenous injections of 50% glucose or even blood transfusions were resorted to. Due to a lowered resistance and perhaps also to the use of a tracheal cannula during operation they were prone to develop pneumonia, and a number were lost from that disease in spite of the use of sulfa drugs. Perhaps the desire to have the animals survive led to undue apprehension, and caused intervention by special feeding when it was unnecessary. It is surprising that in spite of a decreased appetite the gain in body weight during gestation (Table 1) was in the normal range. Gardner and Allen (1942) report a normal gain in weight in pregnant hypophysectomized mice, and Knobil and Caton (1953) state there is a comparable gain in body weight in hypophysectomized and normal rats.

Table 2 shows that 10 of the monkeys hypophysectomized during gestation either aborted or they delivered stillborn infants soon after being operated upon. The percentage of abortions would be reduced if animal K in which pregnancy was not certain was excluded. It is the experience of the writer that a diagonsis of pregnancy by palpation of the uterus during the first month is uncertain in some cases. A laparotomy seemed inadvisable because of added trauma. Menstruation may have been mistaken for the placental sign. They are sometimes very similar for both vary in the extent and duration of bleeding.

It is obvious that in five of the animals (*L*, *M*, *N*, *O*, *P*) abortion was not due to operative trauma for periods of 23-51 days elapsed between hypophysectomy and abortion. These animals were healthy and no reason was apparent why a miscarriage should have occurred. In 2 animals (*Q*, *R*) abortion occurred within a week. In 2 other animals (*S*, *T*) hypophysectomized late in pregnancy still births occurred after periods of 4 and 10 days. The trauma incident to hypophysectomy may have been a causative or at least a contributing factor in terminating pregnancy in these 4 animals.

Thus of the 10 animals listed in Table 2, five definitely known to be pregnant aborted under circumstances in which no causative factor is evident other than the removal of the hypophysis and the normal incidence of abortion.

We have not allowed a sufficient number of intact pregnant animals to go to term to permit an estimate of the frequency of abortion in our colony. As will be shown under "Discussion" the frequency from the data of Hart-

man is high. We have had 3 abortions and 1 stillbirth in intact monkeys. The abortions were on days 49, 63 and 85. The 3 animals which aborted had been scheduled for hypophysectomy but it was postponed. If they had been operated on their abortion would have been attributed to the removal of the hypophysis. The intact animal which aborted on day 85 became pregnant a second time and was then hypophysectomized on day 146. She delivered a stillborn infant (448 gm.) on day 174.[3]

### THE BABIES

The 10 babies which developed to term (Table 1) ranged in weight from 230 gm. to 543 gm. with an average of 390.2 gm. The one which was much underweight (230 gm.) was delivered by caesarean section when the mother was having difficulty in labor. It was lively and took milk from a pipette but was autopsied 5 hours postpartum in order to secure organ weights. The mother was in poor condition throughout gestation, had been repeatedly fed with a stomach tube, and had had occasional intravenous injections of glucose solution and of whole blood. The 8 babies in which sitting height was obtained averaged 190.6 mm.

All the living babies were healthy, active and had a normal coat of hair.

### DISCUSSION

In the preceding section it has been shown that after complete removal of the intrasellar components of the pituitary gland pregnancy continued to term in 10 of 20 monkeys, 6 viable babies being obtained. Of the 4 nonliving babies 2 died during delivery (breech presentation), and 2 were stillbirths, 1 of these being delivered after normal labor and 1, recently dead, being delivered by caesarean section. This gives a ratio of still births to 1 to 5. In the 39 dated pregnancies of Hartman (1932) there were 5 stillbirths (4 marked in his Table 8 and 1 mentioned in the text but not given in the table). This is a ratio of 1 to 7.6. Thus, the incidence of stillborn infants is higher in hypophysectomized than in intact animals. The gestation periods (151–191 days) were in the normal range except in the last case. Hartman (1932) states that gestation varies in length from 146–180 days with an average of 164 days; van Wagenen and Newton (1943) give 147–182 days. Hypophysectomy, therefore, does not legthen the gestation period as is commonly the case in rats (Pencharz and Long, 1933).

In 5 of the 9 monkeys which aborted, pregnancy continued for periods of 23–58 days after hypophysectomy so that it is unlikely that anaesthesia

[3] With our limited colony of about 45 mature females and 4 males it was not possible to attempt a series of sham operations. This would have required a series as large as the hypophysectomized one. In the one sham operation done on the 167th day of pregnancy a macerated fetus was removed by caesarean section on day 208.

or trauma contributed to the abortion. In 4 others, however, the early occurrence of abortion, or still births makes it not improbable that factors other than the actual loss of the hypophysis contributed to the termination of pregnancy. If these 4 are not included it gives an abortion rate of 1 to 3. Hartman (1932) lists 7 abortions in 38 dated pregnancies or a ratio of 1 to 5.4. The frequency of abortion, although high in normal intact monkeys, obviously is still higher after hypophysectomy.

The earliest time in gestation at which the pituitary gland can be removed in monkeys without interrupting pregnancy cannot be definitely determined from our data. It is relatively much earlier, however, than in any other form reported. In rats and mice hypophysectomy before midpregnancy invariably leads to resorption, and the period at which pregnancy is reported to continue after hypophysectomy in other forms is still later. A perusal of Tables 1 and 2 shows that the animal hypophysectomized on day $27 \pm 2$, although probably pregnant, left no evidence of abortion such as blood or abortus in the cage. Of the 4 animals hypophysectomized between days 29 and 34, 1 went to term and 3 did not abort for several weeks. It seems probable that by day 29 or 30, or perhaps slightly later, pregnancy will not be interrupted by pituitary ablation. The difficulty of determining an earliest date had been commented upon by Hartman and Corner (1947) in their experiments on extirpation of the corpus luteum. Monkeys, especially pregnant ones, have to be used more sparingly than pregnant rats and mice.

At the 29th day of pregnancy the monkey embryo measures 7.4 mm. in length, corresponding to a human embryo of the 5th week (Wislocki and Hartman, 1929). The uterus in the specimen studied by these investigators measured $31 \times 37 \times 27.5$ mm. The myometrium and lamina basalis each were 2 mm. in thickness. The placenta, composed of the primary and secondary discs, formed an almost continuous layer only 2 mm. in thickness. The amount of placental tissue at the 29th day thus is rather small. By the 35th day the fetal placenta has almost doubled in thickness (Wislocki and Streeter, 1938) and, due to the increase in size of the uterus, the mass of the placenta has much more than doubled.

It is known that the placenta in monkeys assumes a secretory function earlier than this 29–35 day period. Hamlett (1937) reported that chorionic gonadotrophin can be detected in the urine by day 19 and that it disappears by day 25. Hartman and Corner (1947) have shown by extirpation of the corpus luteum that the placenta by day 25 secretes sufficient progesterone to maintain pregnancy. Dorfman and van Wagenen (1941) report a definite increase in the excretion of estrogens between the 30th and 60th day, the highest level obtained being a 46-fold increase over that in nonpregnant monkeys. They attribute the greater part of the estrogen

secretion to the placenta. The secretion definitely is not from the embryo for its removal, leaving the placenta intact, produced no diminution in estrogen excretion (Dorfman and van Wagenen, 1941) or in the intense sex coloration characteristic of pregnancy (van Wagenen and Newton, 1943). We have pointed out that the sex coloration is not diminished after hypophysectomy during gestation which indicates that after hypophysectomy the placental secretion of estrogen is maintained. Progesterone secretion by the placenta, it seems must likewise be maintained else abortion would occur. Since the placenta continues to secrete these hormones in monkeys after hypophysectomy it appears that this organ in its secretory as in its nutritive role needs no hormone from the hypophysis in order to perform these functions. It requires no stimulating hormones of hypophyseal origin in order to secrete estrogen or progesterone (or ACTH, Smith, 1954). The embryonic or fetal hypophysis is not sufficiently developed, at least in the early stages, to supply trophic hormones.

Hartman (1932) gives the average weight of 22 viable monkey babies as 453 gm., with a range of 330 to 550 gm. Schultz (1937) gives an average weight of 24 babies as 435 gm. and the sitting height as 189 mm. Van Wagenen (1950) states that full-term surviving infants weigh 300–670 gm. at birth and measure 174–224 mm. in body length. Although the average weight reported here is below that given by the above investigators, the weights, except that of the lightest one (230 gm.) come within the normal range. The average of the sitting height is almost identical with that given by Schultz (1937). Only one, *C*, is below the range (168 to 207 mm.) given by Schultz (1933). Knobil and Caton (1953) report that in rats, hypophysectomized during pregnancy, the weight of the fetuses on the 21st day was significantly less than in the controls and that this lower weight was not due to a decreased food intake for the amount of food eaten by the controls was restricted to the amount eaten by the operated animals. The lower average weight of the babies from hypophysectomized monkeys thus is in accord with the findings of these investigators.

Although the lightest baby has the shortest gestation period (159 days), nevertheless, there does not appear to be a correlation between size of offspring and length of gestation. Nor is there a relationship between weight and the stage in pregnancy at which the pituitary gland was removed. The length of the posthypophysectomy antepartum period appears not to influence the development of the infant.

Extensive studies have shown the similarity of the reproductive processes in rhesus monkeys to those in man. The reproductive tract of the female is a replica, in miniature, of that of the human. The length of the menstrual cycle and the structural changes in the uterus during the cycle and during gestation are strikingly similar to those in the human. The

secretory activity of the placenta is also comparable. Its secretion, as in the human, will substitute for those of the ovaries as demonstrated by ovariectomies. Since, as shown in this paper, pregnancy will continue in rhesus monkeys after destruction of the hypophysis, it seeems justifiable to suggest that in man destruction of the hypophysis during gestation by thrombosis, infarction or other processes would not necessarily terminate pregnancy. It seems not unlikely that such a destruction might not become evident until difficulties in labor and the subsequent development of Simmond's disease revealed that apituitarism existed.

## SUMMARY

The results of the complete removal of the intrasellar components of the hypophysis (hypophysectomy) in 20 pregnant rhesus monkeys are reported.

The hypophysectomies were done between the 27th and 156th day after conception ±2 or in some cases 3 days. Ten of the animals went to term. Six living babies were delivered, in 2 cases by caesarean section. Of the 4 nonliving babies, 2 with breech presentations died during birth, and 2 were stillborn after posthypophysectomy periods in excess of 30 days.

The length of gestation was normal except in one case.

Most of the mothers became exhausted either during or by the time parturition was completed.

The posthypophysectomy-antepartum period varied in general with the stage of pregnancy at operation. The longest period was 131 days or nearly five sixths of the gestation period; the shortest was 20 days.

The behavior of the hypophysectomized mothers was abnormal in that they did not eat the afterbirth or cut the cord except in 1 case. Their protective instinct towards their young was normal.

Lactation was of brief duration.

The living babies were healthy and vigorous. Sitting height was normal. Their weights were within the range reported by other investigators, except in 1 case, but their average weight was below normal.

Five animals aborted 23 to 58 days after hypophysectomy. In one the time of abortion was not definitely determined. Two operated upon on days 66 and 101 of pregnancy aborted within a week, and 2 operated upon on days 142 and 156 had stillbirths after 10 and 4 days, respectively.

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# THE EFFECT OF METABOLITES OF CHLORAMPHENICOL (CHLOROMYCETIN)[1] ON THE THYROID OF THE RAT

R. Q. THOMPSON,[2] M. STURTEVANT, O. D. BIRD AND A. J. GLAZKO

*Research Laboratories, Parke, Davis and Company, Detroit, Michigan*

THE metabolic degradation of chloramphenicol has been investigated in bacteria, the rat, guinea pig, dog and man. Following the administration of chloramphenicol to man, the antibiotic is rapidly absorbed and excreted in the urine. A small amount of the antibiotic is excreted unchanged (Ley *et al.*, 1948), but the principal excretory product in man is the conjugate with glucuronic acid, which has no microbiological activity (Glazko *et al.*, 1950). A similar excretion pattern was found in lower animals following oral as well as intravenous administration, but the recovery of total nitro compounds in the urine was considerably less than that found in man (Glazko *et al.*, 1949b). In the case of the rat and guinea pig, a large percentage of the administered dose, following initial absorption, is excreted as the glucuronide via the bile into the intestinal tract. The intestinal flora hydrolyzes the glucuronide and reduces the chloramphenicol moiety to aryl amines which may be partly reabsorbed and excreted in the urine and feces (Glazko *et al.*, 1949b, 1952). It has also been found that enzyme systems present in the tissues of rats and guinea pigs can reduce the nitro group to form primary aromatic amines (Glazko *et al.*, 1949b). Following the administration of a single dose of the antibiotic, significant tissue levels of aryl amines are found in the rat and guinea pig. No increased urinary excretion of aryl amines was found in man following single dose administration. Although large quantities of aryl amines are excreted in the feces of the dog and lower animals, only traces of the administered dose were found as aryl amines and nitro compounds in the stool of man over a 50 hour collection period. Glazko *et al.* (1952) have estimated that in man less than 3% of an administered dose of chloramphenicol is excreted via the bile into the intestinal tract. Therefore, a significant quantity of aryl amines would not be expected to appear either in the urine or stool, since the majority of the dose is rapidly absorbed and excreted in the urine.

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[1] Parke, Davis and Company trademark for chloramphenicol.

[2] Present address: Department of Agricultural and Biological Chemistry, The Pennsylvania State University, State College, Pennsylvania.

In the course of investigating the effects of relatively large amounts of chloramphenicol fed to rats, decreased basal metabolic rate was observed during the study and thyroid hypertrophy was found at autopsy. Recently, Calesnick *et al.* (1954) have indicated that aureomycin and penicillin may disturb normal thyroid functioning by interfering with uracil metabolism and nucleic acid synthesis. Ginoulhiac, (1951) showed that small doses of chloramphenicol protected young rats from the toxicity of administered thyroxine. The following experiments illustrate that the goitrogenic activity of chloramphenicol is not due to the antibiotic *per se*, but to aryl amine metabolities of chloramphenicol which are formed in considerable quantity by certain animal species.

## METHODS AND MATERIALS

Weanling male rats (40–60 gm.) of an albino strain were housed individually in suspended wire mesh cages. All animals received food and water *ad libitum*.

Three different diets were employed in these studies. The *Breeders' diet* contained the following percentages of ingredients: ground corn 33, whole milk powder 21, alfalfa leaf meal 2, whole liver powder 2, Strain G yeast 3, whole wheat flour 31, calcium carbonate 0.5 and iodized salt 0.5. The composition of the *high-iodide purified diet* (92762) is presented in Table 1. The vitamin mix, which was diluted with cerelose, supplied the following amounts of vitamins in mg. per kg. of finished diet: thiamine 8, riboflavin 8, pyridoxine 8, niacin 100, p-aminobenzoic acid 100, inositol 100, pteroylglutamic acid 10, menadione 5. In addition the diet was supplemented with the following vitamins in mg. per kg. of diet: sodium pantothenate 100, biotin 0.1 and vitamin $B_{12}$ 0.03. The following amounts of vitamins A, D and E were supplied respectively per kg. in the portion of oil added to the ration: 90,000 U.S.P. units, 9,000 U.S.P. units and 120 mg. $\alpha$-tocopheryl-acetate. The composition of the *low-iodide diet* (91202) differed from the high-iodide purified diet only in that the potassium iodide contained in the Jones and Foster (1942) salt mixture (31.6 mg. per kg. of diet) was omitted. Both the Breeders' diet and diet 91202 (low-iodide) were estimated to contain approximately 50–75 $\mu$g. of iodide per 100 gm. Because of the relatively large addition of potassium iodide to diet 92762, the content of iodine in this diet was increased to approximately 2,500 $\mu$g. per 100 gm. Therefore, the high-iodide diet contained on the order of 50 times as much iodine as did the other two diets.

D(−)threo-2-dichloracetamido-1-p-nitrophenyl-1,3-propanediol (chloramphenicol) was thoroughly mixed in the diet at the respective levels indicated in the experiments.

TABLE 1. COMPOSITION OF THE HIGH-IODIDE PURIFIED DIET (92762)

| Ingredient | % |
|---|---|
| Casein (hot alcohol extracted) | 25.0 |
| Cerelose | 60.3 |
| Corn oil | 9.85 |
| Jones and Foster salt mix (1942) | 4.0 |
| Vitamin mix | 0.5 |
| Choline chloride | 0.2 |
| Vitamins A, D, E, oil | 0.15 |

Standard U.S.P. desiccated thyroid was also incorporated in the diet in certain of the experiments.

Weanling rats were assigned to the experimental groups so as to obtain equal distribution of body weight. The rats were weighed at least once weekly. At the termination of an experiment, the animals were sacrificed by ether anesthesia, the thyroids were excised, dissected free of fat and connective tissue and weighed either individually or as a pooled group. The method of Astwood and Bissell (1944) was used to determine thyroidal iodine; the method of Glazko *et al.* (1949a) to determine blood levels of aryl amines and nitro compounds.

## RESULTS

### *Effects of Short Term Chloramphenicol Treatment*

In the first experiment, four groups of weanling rats were maintained for 28 days on the high-iodide diet supplemented with 0%, 0.25%, 0.5% and 1.0% chloramphenicol, respectively. The data presented in Table 2 show that greater retardation of growth occurred with increased concentrations of chloramphenicol in the diet. It has been observed in feeding studies that the decrease in growth is proportional to the reduction in food intake at the respective levels of the antibiotic. Additional observations

TABLE 2. EFFECT OF CHLORAMPHENICOL AND DESICCATED THYROID ON GROWTH, THYROID WEIGHT AND THYROID IODINE CONTENT OF RATS MAINTAINED ON THE HIGH-IODIDE PURIFIED DIET

| Treatment | Survival | Weight gain | | Thyroid weight[1] | | Iodine[1] |
|---|---|---|---|---|---|---|
| | | 28 da. | 91 da. | Per rat | Per 100 gm. body wt. | Per 100 gm. thyroid |
| | | gm. | gm. | mg. | mg. | mg. |
| *Experiment 1* | | | | | | |
| 1. Basal diet 92762 | 10/10 | 173 | — | 18.7 (10) | 8.5 | — |
| 2. Chloramphenicol 0.25% | 10/10 | 135 | — | 23.9 (10) | 13.0 | — |
| 3. Chloramphenicol 0.5% | 10/10 | 109 | — | 24.9 (10) | 16.8 | — |
| 4. Chloramphenicol 1.0% | 10/10 | 82 | — | 27.9 (10) | 21.5 | — |
| *Experiment 2—No Desiccated thyroid* | | | | | | |
| 1. Basal diet 92762 | 7/10 | 182 | 404 | 19.8 (4) | 4.3 | 170 (3) |
| 2. Chloramphenicol 0.25% | 10/10 | 121 | 224 | 41.0 (7) | 15.2 | 11.2 (3) |
| 3. Chloramphenicol 0.5% | 10/10 | 109 | 186 | 39.3 (7) | 17.0 | 15.2 (3) |
| 4. Chloramphenicol 1.0% | 10/10 | 89 | 130 | 37.1 (7) | 21.1 | 12.7 (3) |
| *0.0125% Desiccated thyroid* | | | | | | |
| 5. Basal diet 92762 | 10/10 | 183 | 381 | 17.9 (7) | 4.2 | — |
| 6. Chloramphenicol 0.25% | 9/10 | 173 | 341 | 48.0 (6) | 12.5 | — |
| 7. Chloramphenicol 0.5% | 10/10 | 132 | 305 | 49.9 (7) | 13.9 | — |
| 8. Chloramphenicol 1.0% | 10/10 | 122 | 260 | 52.1 (7) | 16.9 | — |
| *0.025% Desiccated thyroid* | | | | | | |
| 9. Basal diet 92762 | 10/10 | 181 | 396 | 25.1 (7) | 5.7 | 127 (3) |
| 10. Chloramphenicol 0.25% | 9/10 | 161 | 341 | 23.3 (6) | 5.9 | 18.3 (3) |
| 11. Chloramphenicol 0.5% | 10/10 | 120 | 291 | 26.6 (7) | 7.7 | 6.4 (3) |
| 12. Chloramphenicol 1.0% | 10/10 | 120 | 291 | 20.0 (7) | 5.7 | 20.8 (3) |

[1] Number in parentheses indicates number of rats sacrificed.

include diarrhea in rats receiving the higher levels of chloramphenicol, as well as the distended cecum which is generally observed in laboratory animals following extensive antibiotic treatment. Mortality of rats even at the 1% level of drug is practically nil.

Although the thyroids in the chloramphenicol-treated rats are larger as compared to the controls, only slight increases in the absolute size of the gland are observed as the antibiotic concentration in the diet is increased. However, if the weight of the thyroid per 100 gm. of body weight is calculated, a more striking difference in the relative gland size is noted among the three treated groups. The data obtained in Experiment 1, indicate that relatively enlarged thyroids which show hyperplasia under microscopic examination are found in rats as result of a high dietary concentration of chloramphenicol.

*Effects of Long Term Thyroid and Chloramphenicol Treatment*

In view of the apparent goitrogenic activity of chloramphenicol, it seemed of interest to determine the effect of this drug on rats supplemented with desiccated thyroid. Two levels of U.S.P. desiccated thyroid, 0.0125% and 0.025%, were added to the high-iodide diet which contained the three respective levels of chloramphenicol as used in Experiment 1. The data obtained in Experiment 2, which lasted 91 days, are also presented in Table 2.

The weight gained by Groups 1–4, which include the control and antibiotic-supplemented animals, was quite comparable at the end of 28 days to the gain made by rats in Experiment 1 receiving comparable treatment. In marked contrast, increased growth is shown by those groups receiving 0.0125% or 0.025% desiccated thyroid in addition to the respective levels of chloramphenicol. The higher level of thyroid promoted no increase in growth above that produced by the lower level of thyroid in those rats receiving 0.25% and 0.5% of the antibiotic, respectively. However, the higher level of thyroid supplement appeared to be some additional benefit to group 12 which received 1% chloramphenicol.

At the end of the experiment, all but three rats in each group were sacrificed, the thyroids were excised and weighed. The 3 rats which were spared in each group were selected so that the average body weight of the remaining rats was comparable to that for the entire group. The rats which were spared were sacrificed a few days later and thyroidal iodine was determined. Since the thyroid weight data for these rats were similar to that obtained for those animals sacrificed at 91 days, it is not included in Table 2.

Although the thyroids from those rats receiving chloramphenicol are larger at 91 days than at 28 days (Experiment 1) there is no gradation in

size due to increasing levels of the drug. On a body weight basis, values for mg. of thyroid per 100 gm. body weight were obtained at 91 days which were quite comparable to those data in Experiment 1, with the exception of the control group whose value would naturally decrease.

The addition of 0.0125% desiccated thyroid did not prevent thyroid hypertrophy in the chloramphenicol-treated rats. It would appear that somewhat larger glands were obtained at the lower level of thyroid as compared to the thyroids from rats receiving no desiccated thyroid. However, on a body weight basis, the relative size of the glands (Groups 6–8) is somewhat less than that found for Groups 2–4, since larger animals were obtained in the thyroid supplemented groups. The higher level of thyroid prevented hypertrophy of the gland over the whole range of chloramphenicol treatment. A level of 0.025% desiccated thyroid would appear to be optimal for promoting growth and preventing thyroid hypertrophy in rats receiving large amounts of the antibiotic.

Since the basal diet contained a high level of iodide, there was a considerable concentration of iodine found in the thyroids of the control animals. It is quite apparent from the data presented in Table 2, that no such increase in thyroidal iodine occurred with chloramphenicol supplementation. However, the thyroidal iodine was not progressively reduced as the level of the antibiotic was increased from 0.25% to 1.0%, nor did the thyroid supplement appear to increase thyroidal iodine.

The growth curves for Groups 1–4 in Experiment 2 have been plotted and are presented in Figure 1. It will be noted that an immediate retardation in the growth rate occurred due to the addition of the antibiotic to the diet. At the end of the 4th week, growth in the chloramphenicol-treated rats had begun to plateau. In view of the preceding findings in regard to hypertrophy of the thyroid, the growth data suggest that the concentration of circulating thyroid hormone in the rat receiving chloramphenicol has diminished at the end of 4 weeks to a level which is no longer optimal for maintaining the initial rate of growth. This interpretation is given support by the observations of Leblond and Eartly (1952) who found that the growth of thyroidectomized rats subsisting on diets low in iodide and thyroxine ceased at the end of 3–4 weeks. However, it is possible that the synthesis and release of thyroid hormone were not completely halted since the rats on chloramphenicol continued to gain slowly. The iodine content of the thyroids from chloramphenicol-treated rats, although considerably less than that found in the controls, was still large in comparison to that found in rats treated for several months with thiouracil (Astwood and Bissell, 1944). Fractionation of thyroidal iodine in glands from chloramphenicol-treated rats has indicated that the majority of the iodine is organically bound.

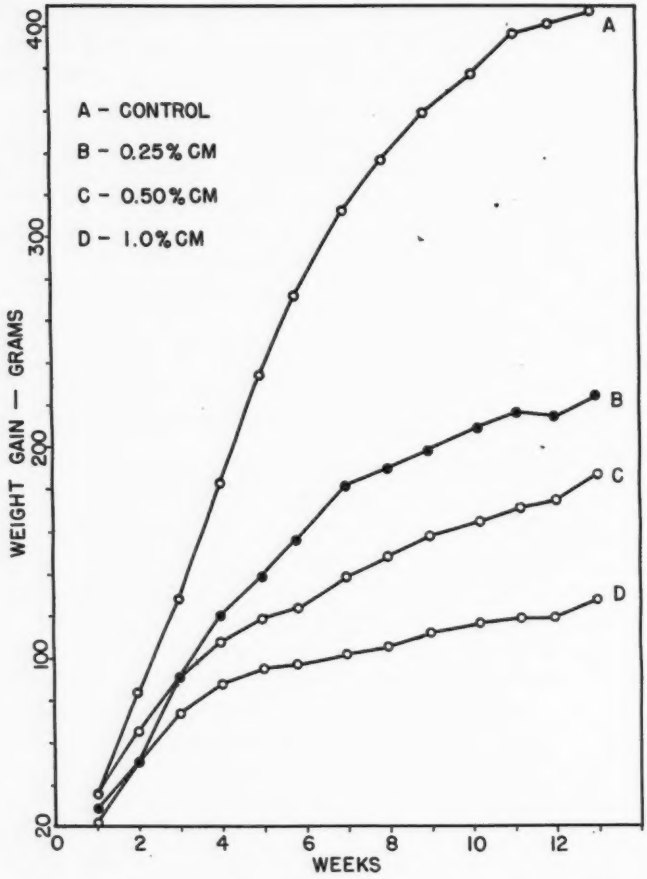


FIG. 1. Effect of chloramphenicol (CM) on the growth of rats maintained on the high-iodide purified diet (92762) in Experiment 2.

*Effects of Thyroid Supplementation Following Chloramphenicol Treatment*

Since desiccated thyroid fed concurrently with chloramphenicol promoted significant growth in Experiment 2, the effect of a thyroid supplement was tested when added to the diet of rats which had received chloramphenicol for 4 weeks. The data obtained in this third experiment are presented graphically in Figure 2. Both the Breeders' diet and the high-iodide diet were supplemented with 1% chloramphenicol and fed to weanling rats. At the end of 28 days, 0.0125% desiccated thyroid was added to both control diets and the drug-supplemented diets. The rate of growth

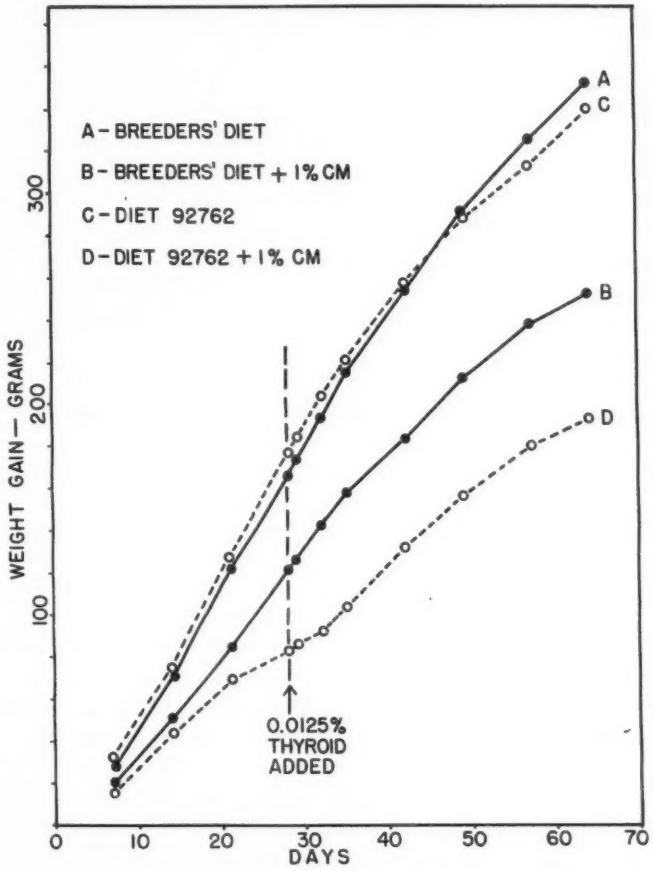


FIG. 2. Effect of the addition of U.S.P. desiccated thyroid in Experiment 3 on the growth of rats maintained on Breeders' diet or the high-iodide purified diet (92762) containing 1% chloramphenicol (CM). Desiccated thyroid (0.0125%) was added to the diet of the respective group on the 28th day; each group contained 10 rats.

was the same on both the control diets and was not affected by the addition of thyroid. As found in the previous experiments, growth of those rats on the high-iodide diet containing the antibiotic began to plateau at the end of 28 days. However, following thyroid supplementation, the growth increased to a rate which was comparable to that occurring during the initial 3 weeks of the experiment. These observations would indicate that the production of the thyroid hormone was being restricted in rats receiving chloramphenicol. As a result the growth rate was retarded, but was immediately restored by exogenous thyroid.

Surprisingly, the growth of rats maintained on the Breeders' diet containing the antibiotic did not plateau at 28 days, nor was the growth affected by thyroid supplementation. Although growth of these antibiotic-treated rats was less than that of their respective controls, it was considerably greater than the growth of rats maintained on the purified diet containing chloramphenicol.

The data given in Figure 2 indicate that the Breeders' diet counteracted much of the growth retarding effect of chloramphenicol. In an attempt to associate this effect with a particular ingredient of the Breeders' diet, the various materials in this diet were incorporated into the high-iodide purified diet. These supplementations did not result in stimulating the growth of chloramphenicol-treated rats.

If the composition of the Breeders' diet is compared with the purified diet, one significant difference assumes importance in experiments concerning thyroid metabolism. The iodide content of the Breeders' diet is low as compared to the purified diet. MacKenzie (1947) has reported that high levels of iodide potentiate the goitrogenic effect of low levels of sulfaguanidine. Therefore, since the dietary levels of iodide could be influencing the goitrogenic activity of chloramphenicol, this effect was further studied in Experiments 4 and 5.

*Effects of Excessive Dietary Iodide on Chloramphenicol-Treated Rats*

In Experiment 4, 1% chloramphenicol was added either alone or concurrently with 0.025% U.S.P. desiccated thyroid to both the high-iodide diet (92762) and to the low-iodide diet (91202), respectively. Data obtained in this experiment are presented in Table 3.

If the data for Groups 1–4 are first considered, it is observed that little difference occurred in the weight gain of the two control groups (1 and 3) either at the end of the 28-day or 64-day experimental period as result of

TABLE 3. EFFECT OF DIETARY IODIDE ON THE GROWTH, THYROID WEIGHT AND THYROID IODINE CONTENT OF RATS MAINTAINED ON PURIFIED DIETS SUPPLEMENTED WITH CHLORAMPHENICOL AND DESICCATED THYROID

| Group | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Basal diet 92762 | + | + | | | + | + | | |
| Basal diet 91202 | | | + | + | | | + | + |
| Chloramphenicol % | | 1 | | 1 | | 1 | | 1 |
| U.S.P.—Desiccated thyroid % | | | | | 0.025 | 0.025 | 0.025 | 0.025 |
| Weight gain[1] | | | | | | | | |
| 28 days | 171 (10) | 125 (9) | 176 (10) | 110 (9) | 165 (10) | 105 (10) | 162 (10) | 128 (10) |
| 64 days | 359 (7) | 195 (6) | 371 (8) | 258 (7) | 356 (10) | 248 (10) | 328 (9) | 259 (10) |
| Thyroid weight[2] | | | | | | | | |
| Excised 73–79 days | | | | | | | | |
| mg. per rat | 20.9 (7) | 39.5 (6) | 32.2 (5) | 55.8 (4) | 14.7 (10) | 13.5 (10) | 17.5 (9) | 19.5 (10) |
| mg. per 100 gm. body weight | 4.8 | 15.7 | 7.3 | 17.1 | 3.5 | 4.4 | 4.3 | 6.0 |
| Thyroid iodine | | | | | | | | |
| mg. per 100 gm. thyroid | 212 | 12.8 | 37.4 | 12.0 | 211 | 54.0 | 63.8 | 37.4 |

[1] Number in parentheses indicates number of rats surviving.
[2] Number in parentheses indicates number of rats sacrificed.

the high dietary intake of iodide. No difference existed at the end of 28 days between the two groups (2 and 4) receiving chloramphenicol. However, Group 4, which was maintained on the low-iodide regimen showed a marked increase in weight gain over Group 2 by the end of the 64-day period. The growth of chloramphenicol-supplemented rats receiving the high-iodide intake began to plateau after 28 days, as has been observed previously, but Group 4 (low-iodide) continued to grow after 28 days at the same initial rate. These data strongly indicate that the level of dietary iodide influenced significantly the growth of rats receiving chloramphenicol.

Morfality was low in this experiment. Only 3 rats died during the test—one each in Groups 2, 4 and 8. However, in Groups 1–4, several rats (3 to 5) in each group were used in additional experiments not pertaining to the work being reported. These rats were selected so as not to disturb the overall average of the group as judged by weight gained. The remaining rats were sacrificed during the 73 to 79-day period, the excised thyroids were weighed and their iodine content was determined.

The weight, both absolute and relative to body weight, of the thyroids from the controls (Group 3) maintained on the low-iodide regimen was about 50% greater than that of the thyroids from the control group (1) receiving diet 92762. The thyroids of both groups (2 and 4) receiving chloramphenicol were about twice as large as the glands of their respective control groups (1 and 3). Of the two chloramphenicol-supplemented groups, those rats maintained on diet 91202 (Group 4) contained thyroids some 40% heavier than Group 2 (diet 92762) but on a body weight basis, the size of the glands for both groups was comparable.

Considerable more iodine was found in the thyroids of the control animals maintained on the high-iodide regimen than in the thyroids of control animals on the low-iodide diet. However, there was no difference between the concentration of thyroidal iodine in the two groups receiving the drug in the two respective diets, although the concentration of thyroidal iodine was greatly reduced below that of the respective controls.

In comparing the data for Groups 5–8 which received 0.025% desiccated thyroid in addition to the respective treatments of Groups 1–4, it is apparent that the thyroid supplement did not affect the growth of the high-iodide control group (5) nor the growth of either group (7 and 8) receiving the low-iodide diet. However, the addition of thyroid to Group 6 which received diet 92762 containing 1% of the antibiotic enabled these rats to grow equally as well as Group 4 which received 1% chloramphenicol in diet 91202. Since the rats in Group 8 grew as well as those in Group 4, the thyroids of the rats maintained on the low-iodide regimen were probably

furnishing sufficient hormone in the presence of chloramphenicol to promote maximal growth under the conditions of the experiment.

The addition of desiccated thyroid at a level of 0.025% resulted in normal-sized thyroids in rats receiving chloramphenicol. Furthermore, in the presence of dietary thyroid, less reduction in thyroidal iodine content occurred in the drug-treated animals. However, this finding was not observed in the other experiments reported in which iodine analyses were performed. The addition of thyroid to the high-iodide diet did not result in an increase in thyroidal iodine in the control rats, as was the case with the low-iodide regimen. It is possible that an iodine level of 211 mg.% represents the io-

TABLE 4. EFFECT OF DIETARY IODIDE ON THE GROWTH, THYROID WEIGHT AND THYROID IODINE CONTENT OF RATS MAINTAINED ON BREEDERS' DIET SUPPLEMENTED WITH CHLORAMPHENICOL AND DESICCATED THYROID

| Group | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Breeders' diet | + | + | + | + | + | + | + | + |
| KI mg./kg. diet | 31.6 | 31.6 | | | 31.6 | 31.6 | | |
| Chloramphenicol % | | 1 | | 1 | | 1 | | 1 |
| U.S.P.—Desiccated thyroid % | | | | | 0.025 | 0.025 | 0.025 | 0.025 |
| Weight gain[1] | | | | | | | | |
| 28 days | 159 (10) | 123 (10) | 154 (10) | 134 (10) | 168 (10) | 148 (10) | 196 (10) | 155 (10) |
| 64 days | 334 (10) | 180 (10) | 353 (9) | 259 (9) | 352 (10) | 273 (10) | 399 (10) | 265 (9) |
| Thyroid weight[2] | | | | | | | | |
| Excised 71–72 days | | | | | | | | |
| mg. per rat | 13.7 (10) | 52.7 (10) | 29.7 (6) | 107 (6) | 15.3 (10) | 33.3 (10) | 23.3 (10) | 32.7 (9) |
| mg. per 100 gm. body wt. | 3.4 | 23.6 | 7.1 | 33.6 | 3.7 | 10.3 | 5.0 | 10.2 |
| Thyroid iodine | | | | | | | | |
| mg. per 100 gm. thyroid | 208 | 6.9 | 49.9 | 7.8 | 181.0 | 10.8 | 70.6 | 6.2 |

[1] Number in parentheses indicates number of rats surviving.
[2] Number in parentheses indicates number of rats sacrificed.

dine saturation point in these thyroids. Thus, further evidence is presented which indicates that the goitrogenic activity of chloramphenicol is potentiated by a high-dietary concentration of iodide. The data would further indicate that this goitrogenic effect is counteracted either by exogenous thyroid or by the removal of much of the dietary iodide.

In Experiment 5, which was conducted simultaneously with Experiment 4, the Breeders' diet was supplemented with KI in an amount equivalent to that added to diet 92762. Additional supplements included 0.025% U.S.P. desiccated thyroid and 1% chloramphenicol. Weanling rats were fed the Breeders' diet *ad libitum* with combinations of the supplements mentioned for a period similar to that in Experiment 4.

The growth data presented in Table 4, show that the addition of KI to the Breeders' diet containing chloramphenicol resulted in a retardation of growth comparable to that observed with the high-iodide diet in Experiment 4. Furthermore, as observed in the preceding experiment, the addition of 0.025% thyroid counteracted the growth-retarding effect of the high level of dietary iodide in the presence of chloramphenicol.

The growth of rats maintained on the Breeders' diet containing 1% chloramphenicol was comparable to that for rats receiving the low-iodide

regimen and was not further increased by thyroid supplementation. The weight and iodine concentration of the thyroids of the control animals in Experiment 5 (Groups 1, 3, 5 and 7) were comparable to those values obtained for the respective control groups in Experiment 4. However, the thyroids from the chloramphenicol-treated groups (2 and 4) which had been maintained on a diet composed of unpurified ingredients in Experiment 5 were considerably larger than those in Groups 2 and 4 of Experiment 4 which had received comparable amounts of dietary iodide and antibiotic in a purified ration. The addition of thyroid did not completely counteract thyroid hypertrophy in the drug-treated rats receiving either the Breeders' diet or the same diet supplemented with iodide, but did tend to produce thyroids of about the same size irrespective of the iodide intake. Mortality was also low in this experiment. Only 3 rats died—one each in Groups 3, 4 and 8. However, 3 rats from Groups 3 and 4, respectively, were not sacrificed for thyroid examination, but were used in other work.

The data from Experiments 4 and 5 show that essentially no difference exists between the Breeders' diet and the purified diet in regard to the amount of growth obtained or the extent of the goitrogenic effect of chloramphenicol if the dietary level of iodide in the purified diet is restricted to an amount comparable to that found in the Breeders' diet.

*Effect of an Amine Degradation Product of Chloramphenicol*

The experiments which have been described, indicate that chloramphenicol, which may be regarded as a p-nitrophenyl derivative, exhibits goitrogenic activity when administered in relatively large amounts to rats for several weeks. This type of compound has not been previously reported to be goitrogenic *per se*. It was of interest to determine if the goitrogenic activity resided in the antibiotic *per se* or in metabolic degradation products formed in the rat.

A number of p-aminophenyl derivatives are known to have goitrogenic activity. Glazko *et al.* (1952) have identified one of the metabolites of chloramphenicol produced in the rat as 1-p-aminophenyl-2-amino-1,3-propanediol. Significant levels of aryl amines are found in the blood of rats receiving chloramphenicol (see Table 6). In view of the possible goitrogenic properties of the reduced free base of chloramphenicol, this compound was administered at a level of 0.1% in the high-iodide diet to weanling rats. After one week of administration the level was increased to 1% of the diet for 6 of the animals. The data obtained in Experiment 6 are presented in Table 5.

Although the growth of the rats and the thyroid weight were affected only slightly, a marked decrease in thyroidal iodine occurred in those rats receiving 1% of the amine in the diet, while the iodine content of the control thyroids increased from 115 mg.% at 7 days to 141 mg.% at 35 days.

TABLE 5. EFFECT OF D(-)-THREO-1-P-AMINO-PHENYL-2-AMINO-1,3-PROPANEDIOL[1] ON GROWTH, THYROID WEIGHT AND THYROID IODINE CONTENT OF RATS MAINTAINED ON THE HIGH-IODIDE PURIFIED DIET

| Treatment | Weight[2] gain | Thyroid weight | | Iodine |
|---|---|---|---|---|
| | | per rat[3] | per 100 gm. body wt. | per 100 gm. thyroid |
| | gm. | mg. | mg. | mg. |
| | | *0–7 days* | | |
| Basal diet 92762 | 40 (20) | 9.0 (3) | 10.1 | 115 |
| 0.1% p-$NH_2$II | 40 (20) | 6.7 (3) | 7.4 | 96 |
| | | *8–14 days* | | |
| Basal diet 92762 | 44 (17) | 9.7 (3) | 6.8 | 123 |
| 0.1% p-$NH_2$II | 40 (11) | 10.0 (3) | 7.0 | 88.5 |
| 1.0% p-$NH_2$II[4] | 36 (6) | 10.7 (3) | 8.0 | 56.2 |
| | | *15–28 days* | | |
| Basal diet 92762 | 107 (14) | 16.7 (3) | 7.2 | 140 |
| 0.1% p-$NH_2$II | 87 (8) | 11.3 (3) | 5.3 | 99.2 |
| 1.0% p-$NH_2$II | 83 (3) | 19.3 (3) | 9.1 | 24 |
| | | *29–35 days* | | |
| Basal diet 92762 | 42 (11) | 14.6 (11) | 5.4 | 141 |
| 0.1% p-$NH_2$II | 49 (5) | 14.4 (5) | 5.5 | 102 |

[1] This compound designated as p-$NH_2$II in Table.
[2] Number in parentheses indicates number of rats included in average.
[3] Number in parentheses indicates number of rats sacrificed.
[4] Six rats transferred from 0.1% to 1% p-$NH_2$II on 8th day of experiment.

Thyroids from rats receiving 1% of the aryl amine contained an iodine concentration of 56 mg.% after one week which further decreased to 24 mg.% at the end of 21 days. Although there was no decrease in thyroidal iodine in rats receiving 0.1% of the amine neither did an increase in thyroidal iodine occur as observed in the controls. Although the reduced free base of chloramphenicol did not cause thyroid hypertrophy in a short-term experi-

TABLE 6. EFFECT OF THE D- AND L-ISOMERS OF CHLORAMPHENICOL ON THE GROWTH, THYROID WEIGHT, THYROID IODINE CONTENT, AND BLOOD LEVELS OF ARYL AMINES AND NITRO COMPOUNDS OF RATS MAINTAINED ON BREEDERS' DIET FOR 6 WEEKS

| Treatment | Weight gain | Thyroid weight | | Iodine | Blood serum | |
|---|---|---|---|---|---|---|
| | | Per rat | Per 100 gm. body wt. | Per 100 gm. thyroid | Aryl $NH_2$[1] | $NO_2$ cpds.[1] |
| | gm. | mg. | mg. | mg. | γ/cc. | γ/cc. |
| Breeders' diet | 258 | 16.0 | 5.1 | 66.2 | — | — |
| Chloramphenicol[2] | 184 | 63.7 | 24.2 | 5.0 | 12 | 11 |
| D-Chloramphenicol[3] | 170 | 54.3 | 22.2 | 3.4 | 14 | 8 |
| L-Chloramphenicol[3] | 188 | 23.7 | 9.1 | 21.3 | 5 | 25 |

[1] Values given as chloramphenicol equivalents. Values obtained for the control rats were subtracted from values found for treated rats.
[2] Obtained by fermentative procedures.
[3] Obtained by chemical synthesis and resolution.

ment, the marked reduction in thyroidal iodine indicates that aryl amine degradation products of chloramphenicol may be potential goitrogens. However, the possible goitrogenic properties of nitro derivatives have not been ruled out.

*Effect of the (L-) and (D-) Isomers of Chloramphenicol*

In Experiment 7, rats of an average initial weight of 65 gm. received at a level of 1% in the Breeders' diet either chloramphenicol obtained by fermentative procedures, the active synthetic D-isomer, or the inactive synthetic L-isomer, respectively. At the end of 6 weeks, thyroid weight and iodine content were determined as well as the blood level of aryl amines and nitro compounds in 3 rats randomly chosen from the respective experimental groups. The data presented in Table 6, indicate that the active D-isomers of chloramphenicol obtained from synthesis or fermentation sources possess similar goitrogenic activity. However, the inactive L-isomer produced only a slight hypertrophy of the thyroid and caused reduction in thyroidal iodine to a much lesser extent as compared to the active isomer. When the blood levels of aryl amines and nitro compounds are compared, one significant difference is observed. The blood level of aryl amines in those rats receiving the inactive L-isomer is only 30–50% as high as found in the blood of rats supplemented with the active D-isomer. On the other hand, the level of nitro compounds is some 2 to 3 times as high in the group receiving the L-isomer. Glazko *et al.* (1954) have found only 20% as much of the inactive L-isomer excreted in the bile of the rat as compared to the active D-isomer of chloramphenicol. This difference in biliary excretion might account for higher blood levels of nitro derivatives from the inactive L-isomer and increased amine formation from reduction of the active D-isomer in the intestinal tract.

In Experiment 7, although the level of nitro compounds was markedly increased by supplementing the diet with the inactive L-isomer of chloramphenicol, the goitrogenic activity was significantly less than that found for the active isomer. Since the blood level of aryl amines was lower in rats treated with the L-isomer, it would appear that the magnitude of the goitrogenic effect of chloramphenicol is dependent upon the extent to which the nitro group is reduced to an amine in the animal.

*Effect of Chloramphenicol and Thyroid on Basal Metabolism*

Early in the experimental work it was noted that rats maintained on chloramphenicol-supplemented diets over long periods of time show definite signs of exophthalmia, as well as enlarged thyroids. Measurements of oxygen consumption were, therefore, undertaken to ascertain the basal metabolic status of these animals. Oxygen consumption was determined on

fasting animals using a modification of the apparatus described by Foster and Sundstroem (1926). Two successive runs were made on each animal, and the volume of oxygen consumed was corrected back to 0° C. Body surface area was estimated from the relationship $S = kw^{2/3}$; where $k = 11.36$, $w$ = body weight in grams, and $S$ = surface area in square centimeters (Carman and Mitchell, 1926). The final results are expressed in terms of ml. $O_2$ consumed per $cm^2$ body area per hour.

Using this procedure, data was obtained on a group of 10 animals maintained on a 2% chloramphenicol diet for 4 months, as well as on a group of 10 control animals. The average oxygen consumption[3] for the two groups was $0.45 \pm 0.05$ ml./$cm^2$/hr. and $0.68 \pm 0.08$ ml./$cm^2$/hr. A second group of 13 animals maintained on a 1% chloramphenicol diet for 7 months showed an average oxygen consumption of $0.45 \pm 0.07$ ml./$cm^2$/hr., as compared with $0.65 \pm 0.09$ ml./$cm^2$/hr. for the corresponding control group. Animals which had been kept on a diet containing 1 and 2% chloramphenicol with the addition of 0.025% desiccated thyroid for 4 months showed normal rates of oxygen consumption.

Similar data were obtained on smaller groups of animals (3 per series) maintained on the high- and low-iodide diets. With 1% chloramphenicol added, the oxygen consumption was 0.55 and 0.69 ml./$cm^2$/hr. for the high- and low-iodide diets respectively, while the corresponding controls were 0.82 and 0.77 ml./$cm^2$/hr.

Since chloramphenicol appears to depress the basal metabolic rate of rats about 30% under the conditions of these experiments, and since the rat is known to have a rather poor temperature-regulating mechanism, it became of interest to determine the body temperatures. Using a group of 7 animals which had been maintained on 2% chloramphenicol diets for 6 months, the average body temperature[3] by rectal thermometer readings $96.7 \pm 1.8$ F°. A corresponding group of control animals showed an average body temperature of $99.2 \pm 1.4$ F°.

## DISCUSSION

The data reported herein show that chloramphenicol exerts a goitrogenic action when administered in relatively large amounts to rats over extended periods. The anti-thyroid effect of chloramphenicol is manifested by enlarged and hyperplastic thyroid glands, reduced thyroidal iodine, decreased growth and a lowered basal metabolic rate. It has been observed in this laboratory that a dietary level of 0.05% chloramphenicol, which would afford an intake approximating the therapeutic dose in man does not exert

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[3] Standard deviation computed from the formula for small numbers of samples: $\sigma = (\Sigma d^2/n-1)^{1/2}$.

any goitrogenic effect in the rat nor is food consumption or growth significantly affected.

The experimental evidence would indicate that aryl amines arising from the degradation of chloramphenicol in the rat are responsible for the goitrogenic activity observed. Although rat and guinea pig tissue preparations have been shown to reduce the nitro group of chloramphenicol, the significance of these reactions in the over-all production of aryl amines is not known. However, it has been definitely established that significant quantities of aryl amines are formed as result of bacterial reduction in the cecum of the rat, and that these products can be absorbed. One specific aryl amine has been identified as a degradation product of chloramphenicol in the rat, but other aryl amines are known to be formed by bacterial reduction (Smith and Worrel, 1950). Since it is well established that compounds of the p-aminophenyl type are goitrogenic when administered to rats in relatively high concentrations, it is not too surprising that p-aminophenyl-1,2-propanediol caused a reduction in thyroidal iodine.

Of particular interest in this problem was the potentiating effect of dietary iodide on the goitrogenic activity of chloramphenicol. It has often been observed that a diet low in iodine will cause thyroid hypertrophy in rats. Similar observations were made during the present experiments in which larger thyroids were obtained from rats maintained on low-dietary iodide as compared to high-dietary iodide. However, despite the goitrogenic activity of chloramphenicol, the relationship in absolute size between the thyroids from antibiotic-treated rats on the two dietary levels of iodide did not change appreciably from that observed in control animals. Nevertheless, the antibiotic-treated rats receiving the low-iodine regimen continued to grow at a greater rate than did those animals receiving the high-dietary level of iodide. Since supplemental thyroid enhanced the growth of the chloramphenicol-treated rats maintained on high-dietary iodide, it would appear that a high intake of iodide potentiates the goitrogenic effect of chloramphenicol.

These observations are difficult to explain. Wolff and Chaikoff (1948) have shown that the *in vivo* incorporation of inorganic iodide into organically bound iodine in the thyroid of the rat is retarded by excessive amounts of inorganic iodide. Recently Fawcett and Kirkwood (1953) presented a theory which may help to explain the potentiating action of inorganic iodide on the goitrogenic activity of aryl amines. Not only do aromatic goitrogens appear to form molecular compounds with iodine in the gland, but the iodide ion also forms a similar type of compound with iodine to produce a triiodide ion. It would therefore be expected that aromatic goitrogens and iodide would compete with tyrosine for the available iodine in the gland.

As result of the observations in the rat concerning the goitrogenic activity of chloramphenicol, the question arises as to the anti-thyroid effect in other species. In a chronic toxicity test in dogs receiving 250 mg. per kg. body weight for 4 months no evidence has been obtained that either hypertrophy or hyperplasia occurs in the thyroid.[4] This is not too surprising in view of the low urinary excretion of aryl amines in the dog following chloramphenicol administration. Schmidt[5] has reported that no gross or microscopic changes were observed in the thyroids of rhesus monkeys which had received 200–800 mg. chloramphenicol per kg. body weight for as long as 90 days. As for man, no reports have appeared in which chloramphenicol has been shown to exert any goitrogenic activity. In view of the limited biliary excretion of the antibiotic in man, significant levels of aryl amines are found neither in the tissues, urine or stools. Consequently, it appears highly unlikely that an aryl amine concentration of the magnitude necessary to exert anti-thyroid activity would accumulate in human subjects.

SUMMARY

1. Chloramphenicol at levels of 0.25–1.0% of the diet has been shown to be goitrogenic in rats when administered over extended periods.

2. Chloramphenicol-treated rats show retarded growth, lowered B.M.R., enlarged and hyperplastic thyroids and reduced thyroidal iodine.

3. The anti-thyroid effect of chloramphenicol is potentiated by excessive dietary iodide. Greater body growth and more normal thyroids are obtained in antibiotic-treated rats if the dietary intake of iodide is restricted or if 0.025% U.S.P. desiccated thyroid is added to the diet.

4. The goitrogenic activity of chloramphenicol appears to be due to the aryl amine degradation products which are formed by bacterial reduction of the antibiotic in the intestinal tract of the rat. One of the identified metabolites, p-aminophenyl-2-amino-1,3-propanediol, when fed to the rat, decreases thyroidal iodine concentration.

5. The significance of these observations as related to the finding of no goitrogenic activity for chloramphenicol in species other than the rat is discussed.

*Acknowledgments*

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[4] Dr. J. K. Weston, Parke, Davis and Company, unpublished observations.

[5] Dr. L. H. Schmidt, The Christ Hospital, Cincinnati, Ohio, personal communication.

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# THE EFFECT OF TESTOSTERONE ON WATER DISTRIBUTION OF SECONDARY SEX GLANDS OF MALE RATS

GUILFORD G. RUDOLPH AND WILLARD R. STARNES[1]

*Radioisotope Unit, Thayer Veterans Administration Hospital and Department of Biochemistry, Vanderbilt University, Nashville, Tennessee*

ALTERATIONS of water distribution have been reported as one of the earliest demonstrable effects of estrogens on the uterus (Talbot, Lowry and Astwood, 1940; Roberts and Szego, 1947) and androgens on the seminal vesicles (Rudolph and Samuels, 1949). The latter reported an increased intracellular water of the seminal vesicles, estimated by chloride space, as early as ten hours after androgen administration to castrated rats. Other changes occurring at this time were increases in $Q_{O_2}$, weight, and fructose content. It was also suggested that changes in organic metabolism preceded the shifts in water and electrolytes and cell growth. In the present study extracellular water was estimated with isotopic sodium in the seminal vesicles and prostates of uncastrated rats and of castrated rats with and without the administration of testosterone.

## METHODS

Male albino rats from Sprague-Dawley Inc. were used in these experiments. When 92 days of age the animals were castrated and twenty-eight days after castration a single subcutaneous injection of 1.0 mg. of testosterone propionate in sesame oil was given to the treated rats. At intervals from 12 to 96 hours after injection the animals were killed. The groups consisted of 5 rats each within a weight range of 380–400 gm. Five normal uncastrated male rats of the same age (120 days at the time of sacrifice) and 5 untreated castrates were used as controls.

The radioactive sodium was supplied as $Na_2CO_3$. Prior to administration it was converted to isotonic NaCl by the addition of HCl.

Two hours before killing each animal one hundred $\mu$c. of sodium$^{24}$ in 1.0 ml. was injected subcutaneously. The injected solution consisted of the isotopic sodium chloride with the necessary quantity of isotonic glucose to make a total volume of 1.0 ml. The rats were anesthetized with ether, and blood collected from the aorta in heparinized syringes. The seminal vesicles and prostates were then excised, blotted, and weighed. Any secretion, if present, was carefully expressed from the seminal vesicles before the glands were weighed. The tissues were then dried to constant weight at 100° C. to determine the water content. The tissues were ashed at 550° C. and the ash transferred to metal counting cups with the aid of water. One drop of 1% Dupanol was added to give

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[1] Present address: Research Laboratory, Veterans Administration Hospital, Birmingham, Alabama.

a uniform film when the samples were dried. The radioactivity was measured with an end window Geiger tube.

The term $(H_2O)_E$ is the extracellular water of the tissue as measured by sodium$^{24}$ according to Manery and Bale (1941). $(H_2O)_I$ was calculated by difference between total water and $(H_2O)_E$.

## RESULTS

In the normal seminal vesicles and prostates the extracellular water was 13.8 and 8.5% respectively. In the untreated castrates, the levels were 37.0% for the seminal vesicles and 31.8% for the prostates (Table 1). The injection of testosterone caused an increase in the sodium space of both glands, $(H_2O)_E$. Twenty-four hours after testosterone injection, the maximum extracellular water, 43.1% for the seminal vesicles and 37.9% for the prostates, was obtained. This was followed by decreases in extracellular water at 48, 72, and 96 hours. The $(H_2O)_E$ of the seminal vesicles at 96 hours was 18.3% which was near the value for normal seminal vesicles. The decrease found in the prostate was not as large with a value of 22.0% at 96 hours.

The wet weights, mg. wet weight per 100 gm. body weight, and percentage of water of the seminal vesicles and prostates are shown in Table 2. While the increase in weight of the seminal vesicles was about 200%, the weight of the prostate increased 180% at 96 hours; however, the seminal vesicles were 72% of normal while the prostate was only 25% of the normal weight. The percentage of water increased at 24 and 48 hours with subsequent decreases at 72 and 96 hours.

The calculated extracellular and intracellular water on an average gland basis is shown in Table 1. Alterations in the extracellular water of the cas-

TABLE 1. RESPONSE OF SEMINAL VESICLES AND PROSTATES TO INJECTIONS OF 1.0 MG. TETOSTERONE PROPIONATE INTO CASTRATED RATS. FIVE ANIMALS IN EACH GROUP

| | Seminal vesicles | | | Prostates | | |
|---|---|---|---|---|---|---|
| Hours after injection | $(H_2O)_E$ gm./100 gm. tissue ± S.E.M. | $(H_2O)_E$* Average gland, mg. | $(H_2O)_I$* Average gland, mg. | $(H_2O)_E$ gm./100 gm. tissue ± S.E.M. | $(H_2O)_E$* Average gland, mg. | $(H_2O)_I$* Average gland, mg. |
| Uncastrated No injection | 13.8±1.7 | 53.2 | 253.5 | 8.52±0.6 | 42.8 | 376.7 |
| 0 No injection | 37.0±0.9 | 36.5 | 37.6 | 31.8 ±2.2 | 14.5 | 22.9 |
| 12 | 37.7±0.9 | 36.7 | 42.2 | 37.4 ±1.5 | 15.2 | 17.6 |
| 24 | 43.1±2.3 | 50.3 | 46.7 | 37.9 ±1.2 | 20.9 | 25.4 |
| 48 | 35.0±0.6 | 59.2 | 80.1 | 30.1 ±0.2 | 22.4 | 41.6 |
| 72 | 30.9±0.9 | 67.8 | 112.1 | 28.1 ±0.3 | 27.5 | 55.4 |
| 96 | 18.3±3.0 | 50.9 | 170.7 | 22.0 ±1.0 | 28.2 | 78.4 |

* $\frac{\text{Average gland weight (mg.)} \times (H_2O)_E}{100} = (H_2O)_E/\text{gland}$

Average gland weight (mg.) ×% $H_2O = (H_2O)$ Total/gland.

$(H_2O)$ Total$-(H_2O)_E = (H_2O)_I$.

TABLE 2. EFFECT OF TESTOSTERONE PROPIONATE ON THE WEIGHT AND WATER CONTENT OF THE SEMINAL VESICLES AND PROSTATES OF CASTRATED RATS. FIVE ANIMALS IN EACH GROUP

| | Seminal vesicles | | | Prostates | | |
|---|---|---|---|---|---|---|
| Hours after injection | Wet weight mg. | Mg. wet weight per 100 gm. body weight | % $H_2O$ | Wet weight, mg. | Mg. wet weight per 100 gm. body weight | % $H_2O$ |
| Uncastrated No injection | 385.9±37.6* | 97.1±9.8 | 79.5±1.4 | 502.5±36.2* | 126.8±11.0 | 83.5±1.0 |
| 0 No injection | 91.4± 3.8 | 23.4±1.2 | 81.0±0.2 | 45.0± 2.7 | 11.7± 0.6 | 82.2±0.8 |
| 12 | 96.9± 3.9 | 25.0±1.1 | 81.4±0.2 | 40.6± 2.8 | 10.4± 0.7 | 80.6±1.9 |
| 24 | 116.6± 5.1 | 30.3±0.7 | 83.1±0.2 | 55.2± 2.1 | 14.3± 0.1 | 83.9±0.7 |
| 48 | 169.2±14.1 | 44.1±1.3 | 82.1±1.0 | 74.5± 5.7 | 19.4± 1.4 | 85.8±0.8 |
| 72 | 219.4± 6.9 | 56.7±2.5 | 81.9±0.1 | 98.0±12.9 | 25.5± 3.9 | 84.4±0.2 |
| 96 | 278.3±24.6 | 66.6±2.7 | 79.5±1.1 | 128.1± 4.2 | 31.0± 2.0 | 83.9±0.4 |

* Standard error of the mean.

trate seminal vesicles from injected rats when calculated on an average gland basis showed increases of up to 100% over castrate controls at 72 hours with a decrease to only 40% over the castrate controls at 96 hours. The $(H_2O)_I$ obtained by difference between total water and extracellular water was increased at each time measured being about 310% over the castrate controls at 96 hours. The $(H_2O)_E$ of normal seminal vesicles is only 65% larger and the $(H_2O)_I$ is 490% larger than the untreated castrates. The extracellular water of the treated prostates progressively increased at each time to a value 96% larger at 96 hours. The intracellular water showed an increase of 230% at 96 hours. The decrease at 12 hours in the intracellular water was probably due to the decreased average weight of the prostates at this time (Tables 1 and 2). The percentage of water also decreased at this time. The $(H_2O)_E$ of normal prostates was 190% over untreated castrates while the intracellular water was 1400%.

## DISCUSSION

The extracellular water (sodium space) of the secondary sex glands of the castrated rat changes with a single injection of testosterone propionate. While the over-all weight of the seminal vesicles increased during the four day period the "sodium space" water increased to a maximum at 72 hours. The $(H_2O)_E$ at this time was larger than that in the mature seminal vesicles. At the same time the mature gland is 75% larger in weight.

Rudolph and Samuels (1949) found that at 20 hours after testosterone propionate administration the "non-sodium space" water of the seminal vesicles increased 142%, compared to only a 12% increase found in the present work. The preparations used in the earlier work were rats castrated at 30 days of age. It is possible that the response of the seminal vesicles

from the rats castrated before puberty is very different from that of the atrophied glands used in the present investigation. The wet weights of the seminal vesicles from rats castrated before puberty differ markedly from the atrophic glands from rats castrated after puberty. The weights of the glands of the present series were about fifteen times larger than the weights of the glands used by Rudolph and Samuels (1949). A second difference is in the proportion of extracellular water. The extracellular and intracellular water was about equal in the untreated rats of the present series while in the glands used in the earlier investigation the extracellular water accounted for about three-fourths of the total water of the seminal vesicles. In the present work any secretion was carefully expressed from the seminal vesicles before the glands were weighed, dried, and counted; however, it was noted that secretion was absent in the glands from the untreated castrates. This is in agreement with the observation of Porter and Melampy (1952). Since the secretion is extracellular and was present in the glands from the treated animals it would result in a high estimate of the "sodium space."

The continued growth of the glands for 96 hours is in agreement with Hays and Mathieson (1945), although the animal preparation used was different. The metabolic response of the seminal vesicles from animals castrated at 60 or 90 days is similar to that of the glands from rats castrated when immature (Rudolph and Starnes, 1954, and Rudolph and Samuels, 1949).

## SUMMARY

The "sodium space" water of the seminal vesicles and prostates of castrated rats increased at 12 and 24 hours after a single injection of testosterone propionate followed by a decrease at 48, 72, and 96 hours. With the growth of the glands the intracellular water increased more than did the extracellular water.

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# THE EFFECT OF ESTRADIOL BENZOATE AND CORN OIL ON BONE STRUCTURE OF GROWING COCKERELS EXPOSED TO VITAMIN-D DEFICIENCY

WALTER LANDAUER

*Storrs Agricultural Experiment Station, University of Connecticut, Storrs*

IN 1934 Kyes and Potter reported the remarkable observation that cyclic changes in ossification of the long bones occur in pigeons parallel with, and indeed dependent upon, the alternations of ovarian activity. This was the first evidence for modifications of the skeleton brought about by estrogens, though it had been known that the serum calcium level may show such a dependence (Riddle and Reinhart, 1926). During the succeeding years extensive experimental work was conducted with mammals and birds relative to the effects of estrogens on ossification.

With fowl tests of this nature were first made by Zondek (1936, 1937) and by Landauer, Pfeiffer, Gardner and Man (1939). A histological account of the changes observed in these experiments was given by Landauer and Zondek (1944). At that time (viz., 1941) an experiment was also conducted to determine the effect, as far as bone structure was concerned, of estrogen in growing cockerels which were kept on a diet deficient in vitamin D. In one of the control groups the animals were injected with oil in order to observe if fat, such as the vehicle in which the estrogen was administered, had any effect. The principal results of these experiments are to be reported very briefly in the present account.

## MATERIAL AND METHODS

Barred cockerels from crosses of Rhode Island Red hens by Barred Plymouth Rock cocks were used for the experiments. They were raised in chick batteries behind window glass, and were fed a complete diet, supplemented with an adequate amount of cod liver oil. At the age of 45 days the animals were distributed into six groups, as follows:

Group I—vitamin-D deficient diet
Group II—vitamin-D deficient diet+500 γ/day estradiol benzoate
Group III—vitamin-D deficient diet+2,000 γ/day estradiol benzoate
Group IV—complete diet+2000 γ/day estradiol benzoate
Group V—complete diet
Group VI—vitamin-D deficient diet+0.25 or 1.0 cc/day corn oil

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As vitamin-D deficient diet we used the high-cereal rachitogenic diet of Nonidez (1928), viz., a mash composed of 38.5% wheat bran and 15.4% each of yellow corn meal, sifted ground oats, white middlings and whole meat scraps. No cod liver oil was added. The estradiol benzoate and the corn oil ("Mazola") were injected once daily intramuscularly. Initially, there were six animals each in groups I and VI, five each in groups II, III and IV, and ten in the untreated control group (V) that was kept on a complete diet. Animals were killed for study 30, 41 and 57 days, respectively, after they had been put on the experimental diet, and at the latter time the experiment was terminated.

## RESULTS

In the light of present knowledge, it is clear that the rachitogenic diet that was used in these tests leaves much to be desired since it is very low in carbohydrates and probably, in addition to vitamin D, is deficient in vitamins A and E. It will be seen, however, that certain conclusions may safely be drawn from our results.

At the end of the test period there were striking differences in gross appearance between animals of some of the experimental groups. The cockerels of group I, on unsupplemented D-deficient diet, were unable to remain standing for more than a few seconds or to hold their wings in a normal position (Figs. 1 and 2). Such hypotonicity of voluntary musculature is well-known in grave cases of rickets. In contrast, animals of groups II and III, which had been injected with estradiol benzoate during the period of their being on the D-deficient diet, showed an entirely normal control of their muscles (Figs. 3 and 4). The same was true for the cockerels of group VI which, while on the D-deficient diet, had received daily injections of oil (Figs. 5 and 6). The latter birds also lacked, of course, the estrogen- (or, in the case of group I, the starvation-) effect on the secondary sexual structures, showing normal comb development.

Changes in body weight of the animals in the different groups are illustrated in Figure 7. The cockerels on the complete diet (group V) made normal gains in weight until close to the termination of the experiment. The birds on the unsupplemented D-deficient diet (group I) increased only slightly in weight. Estrogen treatment on normal diet (group IV) produced a retardation in body size as was first shown by Zondek (1936). What is of interest here, however, is the fact that, as compared with the animals on unsupplemented D-deficient diet, those receiving in addition daily injections of estrogen (groups II and III) or corn oil (group VI) showed a marked improvement in body weight gains.

Weight of the parathyroid glands relative to the body (Table 1) was much, and to about the same extent, increased in the animals of groups I,

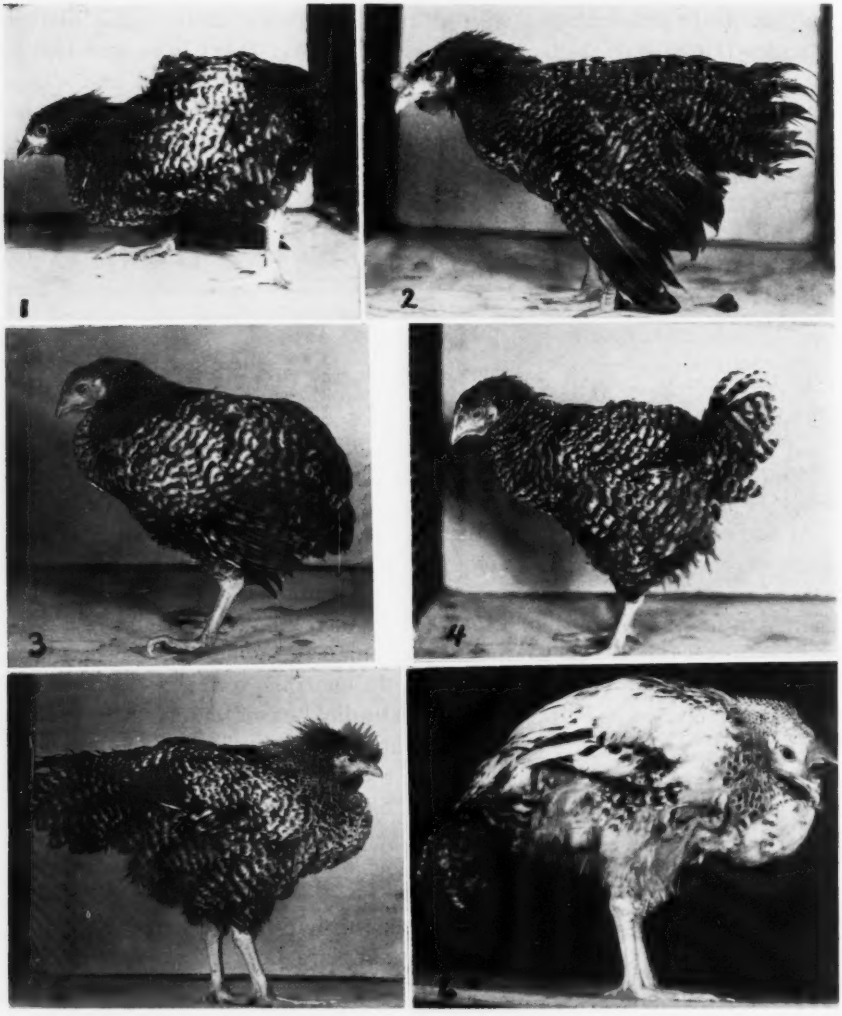


FIG. 1. ♂223, on D-deficient diet for 50 days (group I).

FIG. 2. ♂181, on D-deficient diet for 50 days (group I).

FIG. 3. ♂221, on D-deficient diet for 50 days and 500 γ/day estradiol benzoate (group II).

FIG. 4. ♂183, on D-deficient diet for 50 days and 2,000 γ/day estradiol benzoate (group III).

FIG. 5. ♂230, on D-deficient diet for 50 days and 0.25 cc./day corn oil (group VI).

FIG. 6. ♂231, on D-deficient diet for 50 days and 1 cc./day corn oil (group VI).

TABLE 1. WEIGHT OF PARATHYROIDS IN MG/100G BODY WEIGHT
(standard errors)

| Group | I | II | III | IV | V | VI |
|---|---|---|---|---|---|---|
| Weight | 11.3±1.54 | 11.3±1.44 | 12.4±1.68 | 5.7±0.47 | 3.01±0.78 | 8.7±0.96 |

II and III, i.e. the administration of estrogen did not modify the parathyroid enlargement which had developed on account of vitamin-D deficiency. In group VI size of the parathyroids was intermediate between that in the untreated control animals and that in the cockerels maintained on D-deficient diet, but the differences between group VI on the one hand and groups I, II and III on the other are not statistically significant.

Macroscopic examination of long bones (humerus, femur, tibia) of those

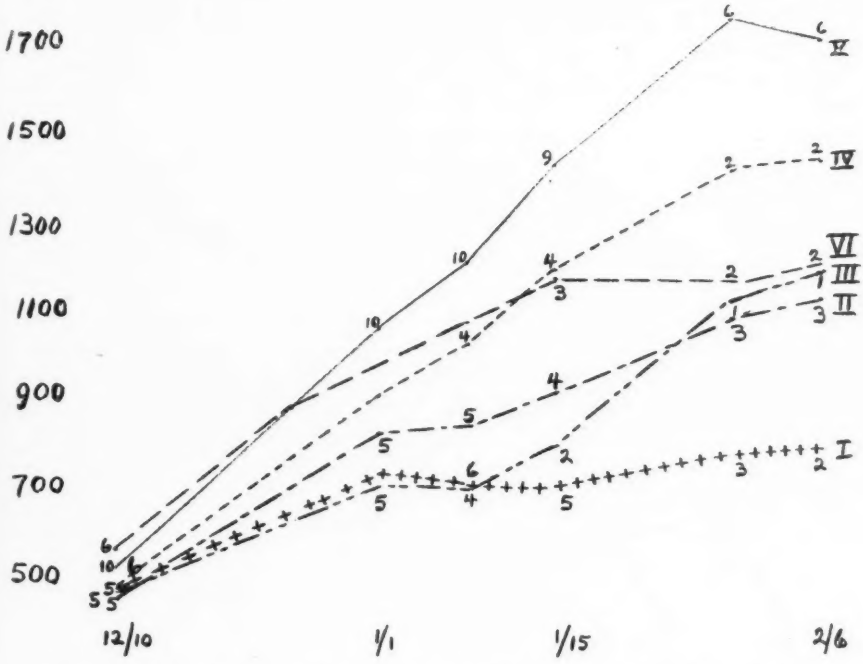


FIG. 7. Changes in body weight of cockerels on a D-deficient diet (I), a D-deficient diet supplemented by either 500 γ/day (II) or 2,000 γ/day (III) estradiol benzoate, a complete diet and injection of 2,000 γ,/day estradiol benzoate (IV), a complete diet without treatment (V), and a D-deficient diet supplemented with either 0.25 cc. or 1 cc./day corn oil (VI). The period shown covers the time from the inception of treatment (age of cockerels 45 days) to the termination of the experiment. The numbers of animals used for each mean of weights is shown along the curves.

birds that were killed at the end of the experiment showed clear differences between the groups. In the frontally split bones it could be seen that the animals of group IV (estrogen and normal diet) had developed the well-known massive hyperossification; in group I (D-deficient diet) the shaft of the long bones was thin and there was much uncalcified tissue in the interior, in extreme cases completely occluding the marrow cavity; the long bones from animals of groups II and III (D-deficient diet and estrogen) did not show gross evidence of osteoporosis of the shaft and the endosteal deviations from the normal appearance were much less obvious than in group I; the bones from animals of group VI ressembled grossly those of groups II and III.

X-ray pictures were taken of the wing and leg bones of all birds, but reference will only be made to those taken at the conclusion of the tests since they showed the most extreme conditions. Among the wing bones (Fig. 8) the humerus is especially instructive. Estrogen treatment on normal diet led to extreme hyperossification. In group I the shaft of the humerus can barely be distinguished and endosteal bone trabeculae are entirely lacking.

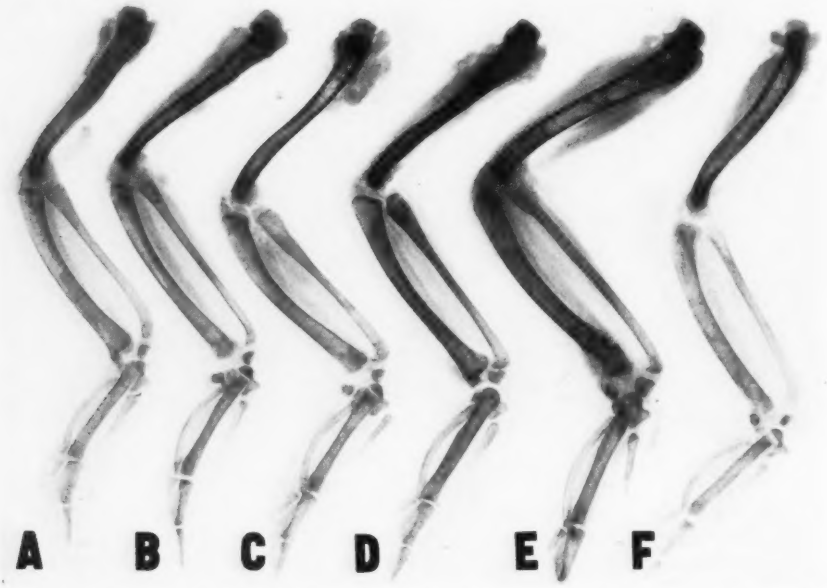


FIG. 8. Radiographs of wing bones of cockerels at the age of 102 days, 57 days after the inception of the experiment. A: ♂181, group I; B: ♂221, group II; C: ♂183, group III; D: ♂209, group IV; E: ♂200, group V; F: ♂230, group VI. ♂230 had received subcutaneous injections of 0.25 cc. corn oil daily while on the vitamin-D deficient diet.

In groups II and III the shaft is relatively well preserved and there are some endosteal bone trabeculae, though fewer than in the untreated controls. In group VI the humerus holds a position intermediate between group I on the one hand and groups II and III on the other, both with reference to preservation of the shaft and development of endosteal bone.

The radiographs of the leg bones showed, on the whole, conditions similar to those observed in the humerus. Cup-shaped and flaring deformation of the proximal end of the tibia was conspicuous in the bones of groups I and II, but not in those of the other groups. Delayed ossification was noticeable in the distal head of the tibia and the proximal one of the tarsometatarsus of animals from groups II, III and, to a lesser extent, group VI.

Histological examination of the long bones of animals in group I showed presence of the typical picture of rickets, in particular excessive formation of osteoid. The bone of the shaft was thinned and there was some fibrous degeneration of marrow. The latter symptoms probably are an expression of concommitant vitamin-A deficiency. There was some osteoid present in the long bones of animals in groups II and III, but much less than in those of group I. There were quantitative differences between the bones of groups II and III. The amount of osteoid was smaller in the bones of group III than in those of group II, and some evidence of beginning hyperossification was found in the marrow cavities of group III bones. The bones of groups II and III showed no signs of osteoporosis of the shaft and, as compared with the bones of group I, there was little fibrous marrow metaplasia. In animals killed between 30 and 57 days after beginning of the experiment gradual changes could be seen in histologic bone structure. In group I the amount of osteoid tissue in the bones steadily increased. The same, on a much lower level, was true for the bones of groups II and III, except that in the bones of those group III animals which had been killed in the earlier stages, no osteoid tissue at all was yet present. In group III signs of hyperossification occurred only toward the end of the experimental period.

As far as group VI was concerned, bone structure in early stages resembled that observed in group II. As the period of treatment continued, the amount of osteoid tissue increased and exceeded in the final stages that observed in bones of groups II and III, though it was in amount much below that found in bones of group I. Toward the end of the experiment the group VI bones also showed some evidence of shaft osteoporosis and formation of fibrous marrow.

## DISCUSSION

The first question with which our experiments were concerned relates to the effect of estrogen treatment on bone structure of birds maintained on a vitamin-D deficient diet, or, to put it antithetically, to the necessity of

adequate amounts of vitamin D being present for estrogen to exert its typical and dramatic effects on structure of the long bones.

Two earlier reports seem to have dealt with this problem. Segaloff and Cahill (1943) found that estrogen-treated mice which were fed a vitamin-D deficient diet showed bone changes (i.e., hyperossification) similar to, if not identical with, those developing in the presence of a complete diet. In view, however, of the well-known refractoriness of mice to nutritional conditions which in other vertebrates produce rickets, the choice of mice as test animals seems peculiarly unfortunate. Saviano (1935), on the other hand, reported on experiments with three groups of rats, one being maintained on the Sherman-Pappenheimer vitamin-D deficient diet, the second, in addition, receiving injections of folliculine (*Progynon* Schering), and the third having its diet supplemented by vitamin D (*Vigantol* Bayer). The animals of the first group remained small and developed florid rickets with its attendant muscular weakness. The rats in the second group grew about as well as those in the third one. Shape and structure of the bones was entirely normal in rats of the third group, whereas the animals in the second group assumed a somewhat intermediate position with reference to shape of epiphyses, width of zone of proliferating cartilage, and so forth. Our own observations are in excellent general agreement with those of Saviano. It may be concluded that in growing fowl and rats estrogen treatment lessens the severity of the bone lesions caused by vitamin-D deficiency. Our tests show, in addition, that acute vitamin-D deficiency suppresses almost entirely the hyperossification and other skeletal events which estrogen initiates under normal conditions. Our results suggest, furthermore, that the beneficial effects of estrogen treatment, as far as rickets is concerned, declined in extent toward the end of the experimental period.

It seems probable that calcium absorption from the intestines plays an important role in the mechanism by which estrogen alleviates the skeletal symptoms of vitamin-D deficiency. In the presence of rickets intestinal calcium absorption is reduced; it is raised by supplementary vitamin-D (Guassardo and Liotta, 1940; Nicolaysen, Eeg-Larsen and Malm, 1953). Estrogen treatment, on the other hand, increases the over-all retention of calcium, both by raising absorption from the intestines and by facilitating calcium deposition in the skeleton (Govaerts, Dallemagne and Melon, 1951; Jowsey, Oliver, Maw and Common, 1953; Common, Maw and Jowsey, 1953). It may be assumed that estrogen treatment corrects to some extent the deficiency of calcium absorption brought about by vitamin-D deficiency.

Another function in which vitamin-D deficiency and estrogen treatment appear to have opposite effects is serum and bone phosphatase activity. In avian (as in mammalian) rickets acid phosphatase activity of the blood

serum is greatly increased (Common, 1936) and the same holds for alkaline bone phosphatase (Combs, Norris and Heuser, 1942). Estrogen treatment, on the contrary, produces a lowering in acid phosphatase activity of the blood serum and in alkaline phosphatase activity of the bones (Day and Follis, 1941; Buchwald and Hudson, 1944). It is not known, however, if these changes in enzyme activity are direct or secondary consequences of the two experimental conditions; nor do we know what is the combined effect of vitamin-D deficiency and estrogen treatment on phosphatase activity.

Great enlargement of the parathyroid glands is a well-established symptom of vitamin-D deficiency. Doyle (1925), Nonidez and Goodale (1927) and Higgins and Sheard (1928) observed this first in poultry, and it is clearly shown by our present data (Table 1). In estrogen-treated ducks Benoit, Clavert and Cabannes (1944) found an initial enlargement and a secondary regression of the parathyroids. Crepax and Moggian (1952) observed that the parathyroids were enlarged in estrogen-treated rats. Our data likewise show a significant increase in parathyroid weight of estrogen-treated cockerels, but this enlargement is not nearly as great as that found in the birds suffering from rickets. In the presence of vitamin-D deficiency the injection of estrogen had no modifying effect on parathyroid weight, i.e. the parathyroids showed the same enlargement as in the non-treated birds with rickets.

It has been reported that fats added, within certain limits, to a normal diet promote bone calcification in rats and mice (French, 1942; Bunkfeldt and Steenbock, 1943; Silberberg and Silberberg, 1950; Silberberg, Opdyke and Silberberg, 1952). It has been found, furthermore, that in rats dietary fat, if not fed in excess amounts, has definitely antirachitic properties and promotes the healing of rickets (Zucker and Barnett, 1923; McDougall, 1938; Jones, 1940; Knudson and Floody, 1940). It is generally accepted that these beneficial effects are brought about by changes in acidity of the intestinal tract with a consequent improvement of calcium retention.

Since the estradiol benzoate, used in our experiments, was dissolved in sesame oil, there was some question as to how much, if any, of the effect of treatment might have been due to administration of supplementary fat. The results with intramuscular injection of 0.25 cc. or 1.0 cc. of corn oil daily (our group VI) indicate that such amounts of fat supplement were equivalent to the estrogen injections in their effects on muscular tonicity and general body growth. When cockerels on vitamin-D deficient diet were treated with corn oil the structure of the long bones, as revealed by X-ray photographs and histological studies, was less abnormal than in the group without supplementary oil. This antirachitic effect of corn oil was, however, much less pronounced than that produced by estrogen injections. The

smaller dose (0.25 cc/day) of corn oil was somewhat more effective than the larger one (1.0 cc/day). Moreover, animals of this group, killed after increasing periods of experimental treatment, showed a trend of lessening protection by corn oil, the animals sacrificed at the termination of the tests exhibiting the most definite symptoms of rickets. Even in this final lot, however, the bone histology was much less abnormal than in the animals on unsupplemented vitamin-D deficient diet.

## SUMMARY

Cockerels which had been raised on a normal diet to the age of 45 days were at that time put on a high-cereal rachitogenic diet without supplementary vitamin D or exposure to ultraviolet light. Two groups received daily intramuscular injections of 500 and 2,000 $\gamma$, respectively, of estradiol benzoate. In another group the animals were given daily intramuscular injections of 0.25 and 1.0 cc. corn oil, respectively. The experimental treatments continued for 57 days.

By present standards (the experiments were performed in 1941) the experimental diet was not satisfactory since it was presumably deficient in vitamins A and E as well as D. The results seem to justify, however, the following conclusions:

1. Estrogen treatment and the injection of corn oil prevented the loss of muscular control which was typical of the birds on the unsupplemented vitamin-D deficient diet.

2. Both estrogen treatment and injected corn oil considerably improved gains in body weight.

3. Estrogen treatment had no effect on the enlargement of the parathyroid glands which occurs as a consequence of rickets. The same is probably true for injection of corn oil. Injection of estrogen into birds which were fed a normal diet led to enlargement of the parathyroids, but this increase in weight was less extreme than that found in birds on a vitamin-D deficient diet.

4. Macroscopic, roentgenologic and histologic examination of long bones showed that estrogen treatment as well as injection of corn oil protected the animals against occurrence of the extreme forms of rickets, but bone structure remained far from normal. The higher dose of estrogen (2,000 $\gamma$/day) was more effective than the lower one (500 $\gamma$/day). The protective value of corn oil declined in the course of the experiment. Even those birds which received the larger amounts of estrogen showed at the end of the experiment only slight indications of hyperossification.

*Acknowledgment*

The estradiol benzoate used in our work was furnished by Ciba Pharmacentical Products, Inc. through the courtesy of Mr. R. S. Mautner.

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# NOTES AND COMMENTS

## EFFECT OF THYROXIN TREATMENT ON THE DISSIMILATION OF DL-ALANINE-1-$C^{14}$ BY KIDNEY AND LIVER HOMOGENATES

Thyroxin at a concentration of $10^{-5}$ M inhibits glutamic acid oxidation by a washed residue of rat kidney cortex homogenate (Feldott and Lardy, 1951). Liver tissue from rats maintained on an iodinated casein-supplemented diet is low in sarcosine oxidase, the enzyme that demethylates sarcosine to formaldehyde and glycine (Swendseid and Swanson, 1952). On the other hand, thyroid feeding is supposed to increase the concentration of D-amino acid oxidase activity of liver, but not of kidney, (Klein, 1939) and thyroidectomy to decrease amino acid oxidase activity of liver, but to increase the activity of the enzyme in kidney if DL-alanine is added to the supernatant of tissue homogenates and oxygen consumption is measured (Cagan Gray and Jensen, 1950). More direct approaches to resolve this conflicting picture are necessary. We would like to report unexpected data which indicate that *DL-thyroxin injection decreases rather than increases dissimilation of DL-alanine-1-$C^{14}$ by liver and perhaps by kidney homogenates*, as evidenced by the evolution of $C^{14}O_2$ (Table 1).

Male rats of the Sprague-Dawley strain, weighing 160 to 200 gm., were used. Immediately after decapitation, 1.5 gm. of tissue per 2 ml. of 0.1 M phosphate buffer (pH 7.4) was minced in a ground glass homogenizer. Two ml. aliquots of the homogenate were placed in a Warburg flask containing 0.05 mg. ($0.6\times10^{-3}$ mc.) DL-alanine-1-$C^{14}$ dissolved in 0.1 ml. distilled water. A filter paper square was inserted into the center well containing 0.2 ml. of 5 per cent KOH. Each flask was oxygenated, then incubated at 37° C. in the Warburg apparatus for 45 minutes. At the end of the incubation, the filter paper was removed from the center well, spread out on a watch glass and counted.

TABLE 1. $C^{14}O_2$ PRODUCTION FROM LABELED DL-ALANINE-1-$C^{14}$ BY TISSUE HOMOGENATES FROM THYROXIN TREATED DATS

| Controls* | Liver Relative Radioactivity of $CO_2$ Produced | Kidney Relative Radioactivity of $CO_2$ Produced |
|---|---|---|
| | 100 | 100 |
| Thyroxin Treated | 62 | 67 |
| | 101 | 50 |
| | 63 | 162 |
| | 35 | 83 |
| | 74 | 83 |
| | 41 | 86 |
| | 46 | 104 |
| | 34 | 100 |

* Controls arbitrarily taken as 100. This activity averaged 2.0% of administered counts eliminated as $C^{14}O_2$ for kidney homogenates and 1.3% of administered counts eliminated as $C^{14}O_2$ for liver homogenates. Results obtained in single animals are given, and in each experiment controls were run. The experimental rats were injected intramuscularly with 0.1 mg. DL-thyroxin per 100 gm. body weight daily for a 10 day period.

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SUMMARY

Thyroxin injection decreases rather than increases dissimilation of DL-alanine-1-$C^{14}$ by liver and perhaps by kidney homogenates.

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CLAUDIA RATLIFF-RAYFORD[1]

*Department of Biology*
*Catholic University of America*
*Washington, D. C.*

[1] Public Health Service Research Fellow of the National Institutes of Health, Bethesda, Maryland.

## COMPARATIVE EFFECTS OF SOMATOTROPHIC HORMONE AND PANCREATIC HYPERGLYCEMIC FACTOR ON BODY WEIGHT AND CHONDROGENESIS IN THE HYPOPHYSECTOMIZED RAT

A relationship between the anterior pituitary somatotrophic hormone (S.T.H.) and the hyperglycemic-glycogenolytic factor of the pancreas (H.G.F.) has been indicated. The suggestion that the growth-promoting effect of S.T.H. is mediated through H.G.F. was advanced recently by Elrick (1953) who presented some supporting evidence to the effect that H.G.F. can produce an increase in width of the proximal epiphyseal cartilage of the tibia in young hypophysectomized rats. We have repeated and extended these experiments on two occasions to show that highly purified H.G.F. is incapable of increasing body weight or epiphyseal cartilage in these animals. Since the present study was undertaken, Geschwind and Staub (1953) also have demonstrated that purified H.G.F. is inactive by the "tibia test."

METHODS

Female Sprague-Dawley rats, hypophysectomized at 26–28 days were maintained for 14 days after operation and divided into groups homogeneous as to weight. In a preliminary experiment H.G.F.[1] and S.T.H.[2] were injected intraperitoneally in saline in single daily dosage of 20 μg. for 15 days. Weight gain and tibial measurements by the method of Greenspan *et al.* (1949) are given in Experiment No. 1, Table 1. In a second experiment the daily dosage of H.G.F.[1] and S.T.H.[2] was 50 μg. After 4 days of treatment approximately half the animals were weighed and sacrificed for tibial measurement and histologic examination,[3] while the remainder were continued for a total of 14 days treatment. Results are given in Experiment No. 2, Table 1. In both experiments control rats received intraperitoneal saline daily.

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[1] Highly purified H.G.F. kindly furnished by Dr. A. Staub of Eli Lilly & Co. as "Glucagon-Lilly" lot 208-108B-234.

[2] Beef S.T.H., Wilhelmi-type, prepared in our laboratory, lot 20-1-2-J51.

[3] We are grateful to Dr. Ernesto Salgado, Institute of Experimental Medicine & Surgery, University of Montreal, for carrying out these examinations.

TABLE 1. EFFECTS OF H.G.F. AND S.T.H. ON BODY WEIGHT AND TIBIAL CARTILAGE WIDTH

| Group | Dosage (μg./day) | Duration | Number of animals | Weight increase (gms.) | Tibial measurement (μ) |
|---|---|---|---|---|---|
| **Experiment No. 1** | | | | | |
| Saline Control | | 15 days | 3 | 11.3 ± 2.3 (S.E.) | 219 ± 13 (S.E.) |
| H.G.F. | 20 | 15 days | 11 | 12.0 ± 1.7 | 195 ± 6 |
| S.T.H. | 20 | 15 days | 6 | 22.3 ± 0.9 | 245 ± 5 |
| **Experiment No. 2** | | | | | |
| Saline Control | | 4 days | 7 | −0.5 ± 1.2 | 150 ± 7 |
| H.G.F. | 50 | 4 days | 8 | 1.5 ± 1.2 | 143 ± 8 |
| S.T.H. | 50 | 4 days | 8 | 5.5 ± 1.5 | 224 ± 15 |
| Saline Control | | 14 days | 9 | 5.1 ± 0.9 | 180 ± 4 |
| H.G.F. | 50 | 14 days | 9 | 5.1 ± 1.8 | 160 ± 1 |
| S.T.H. | 50 | 14 days | 8 | 19.1 ± 1.2 | 257 ± 6 |

## RESULTS

Although Experiment No. 1 gave no intimation that H.G.F. has growth promoting activity the results (with these small groups) were inconclusive in view of the appreciable weight increase in the controls themselves and a somewhat larger response to 20 μg. S.T.H. than we usually obtain with this preparation. Autopsy gave no sign of incomplete hypophysectomy.

However, from the results of Experiment No. 2 it is apparent that highly purified H.G.F. exerts no growth promoting action comparable to S.T.H. Likewise, histologic examination of the tibias gave no evidence of growth stimulation such as that found in the S.T.H.-treated animals.

## CONCLUSIONS

Highly purified pancreatic hyperglycemic factor in dosage of 20 and 50 μg. daily intraperitoneally for as long as 2 weeks exerts no growth promoting action in the young hypophysectomized rat as measured either by body weight or width of tibial epiphyseal cartilage. Pituitary somatotrophic hormone under these conditions produces highly significant responses.

Although some evidence indicates that S.T.H. stimulates the pancreatic alpha cells to secrete hyperglycemic factor, it is not apparent from these experiments that the growth promoting action of S.T.H. is mediated in this way.

L. MITCHELL, W. B. RICE and R. GIRERD

*Research Laboratories*
*Frank W. Horner Limited*
*5485 Ferrier Street*
*Montreal, Canada*

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## THE REMOVAL OF CIRCULATING ANTIDIURETIC HORMONE BY THE KIDNEY

Recently Ginsberg and Heller (1953) have called attention to the rapidity and sites of removal from the circulation of injected posterior pituitary extract. Prior to the date of their publication experiments of a similar nature, though more limited in scope, had been carried out in this laboratory. The results of the latter studies may merit brief publication for their confirmatory value.

### METHODS

The disappearance of pitressin from the circulation was studied in two groups of 300 gm. white rats. The rats of one group were anaesthetized with ether. Through a long, midline abdominal incision the kidney pedicles were exposed one after the other by gentle retraction of the viscera toward the opposite side. A curved, noncutting needle was used to pass a #40 cotton thread around each renal pedicle and the kidney removed from the circulation by a tight knot. The rats of the second group were sham operated. The surgery required 5 to 7 minutes' time.

After preparation as described, pitressin in various doses diluted with saline to a total volume of 0.5 ml. was injected into a vein of the dorsum of the hind foot. Representative animals of each group were given control injections of 0.5 ml. of isotonic saline containing no pitressin. Exactly 5 minutes after the injection, 3 to 6 ml. of blood was rapidly withdrawn from the abdominal aorta and the animal sacrificed. The serum was separated by immediate centrifugation and promptly assayed in 6 test animals for its antidiuretic hormone activity by a method previously described (Crawford and Pinkham, 1954).

Calculations of recovery of the injected pitressin were made according to the same assumptions regarding blood volume and plasma partition as used by Ginsburg and Heller (1953).

### RESULTS AND DISCUSSION

The results are shown in Table 1. The number of experiments at any given dose level was small but recoveries were consistent at the various levels when expressed as per cent of administered dose. Average recovery for the sham operated animals was 3.7% of the pitressin given. This is comparable to the five-minute recovery reported by Ginsberg and Heller (1953) of 1.7%. The importance of the kidney in pitressin clearance is evidenced by the average recovery of 46% of the injected material from animals in which the kidneys had been excluded from the circulation. This figure is of the same order but again higher than that (17.7%) reported by Ginsberg and Heller (1953).

TABLE 1. RECOVERY OF PITRESSIN 5 MINUTES AFTER INTRAVENOUS INJECTION IN SHAM OPERATED RATS AND RATS WITH THE KIDNEYS EXCLUDED FROM THE CIRCULATION

| | Recoveries (per cent administered dose) | | | | | |
|---|---|---|---|---|---|---|
| Pitressin dose (mU): | 0 | 15 | 50 | 100 | 200 | 300 |
| Sham operated | * | 4.0 | 3.7 | 2.5 | 3.2 | 5.0 |
| Kidneys excluded | † | 53 | 49 | — | — | 37 |

* No detectable A.D.H. activity; (<0.1 mU./1 ml. blood).
† A.D.H. activity = 0.25 mU./1 ml. blood.

Significant antidiuretic hormone activity was found present in the serum of animals in which the kidney pedicles had been ligated and a control injection of 0.5 ml. of 0.85%

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NaCl given 5 minutes before blood sampling. This was a consistent finding in 4 experiments and is in contrast to the observation of no activity in serum of sham operated animals similarly injected. It is probably an indication of rapid secretion of antidiuretic hormone into the circulation under conditions where its normal removal by the target organ has been blocked. The tendency for "recoveries" of pitressin in these animals to become lesser as the administered dose was increased may be an artifact related to the same phenomenon.

These observations suggest that difficulties in assay of antidiuretic hormone titres in blood taken under physiologic conditions are due to the very rapid removal of extraordinarily large amounts of the hormone by the kidneys and, as Ginsburg and Heller (1953) have shown, by the organs of the splanchnic circulation. As a corollary, it may well be that the most sensitive procedure of assay of anti-diuretic hormone secretion under such circumstances is measurement in the urine of the individual concerned of the ratio of water to total, osmotically effective solute.

J. D. Crawford and Barbara Pinkham

*From the Department of Pediatrics*
*Harvard Medical School and*
*The Children's Medical Service*
*Massachusetts General Hospital*
*Boston, Massachusetts*

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## EFFECTS OF PROLONGED CORTISONE ADMINISTRATION TO HYPOPHYSECTOMIZED RATS

A systematic account of the effects of adrenal oversecretion following the administration of ACTH to hypophysectomized rats has been published by Asling, Reinhardt and Li (1951). Since there is always some doubt about the purity of pituitary hormones, it was considered desirable to repeat this experiment using cortisone instead of ACTH.

### METHOD

Thirty-five male Wistar strain rats were hypophysectomized when they had reached the weight of 50 to 84 gm., average 66 gm. On the following day the experiment was begun. The rats were maintained in individual cages with raised screen floors (room temperature 76 ± 2° F). Food and water was supplied *ad libitum*. The diet consisted of (per 100 gm.): sucrose, 50; casein,[1] 22; whole liver powder,[2] 10; soybean oil, 10; cellulose,[3] 4; and salt mixture,[4] 4. The following micronutrients were added to the diet in place of sucrose (mg./kg. of diet): thiamin hydrochloride, 10; riboflavin, 20; pyridoxine hydrochloride, 10; calcium pantothenate, 60; niacin, 40; 2 methyl 1, 4 naphthoquinone, 5; folic acid, 4; biotin, 0.4; para-

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[1] Extracted, Genatosan Ltd., Loughborough.

[2] Fat-extracted, desiccated, whole liver powder, Silas & Co., Los Angeles.

[3] Solka Floc, grade BW-100, Brown & Co., New York.

[4] U.S.P. XIV.

aminobenzoic acid, 500; inositol, 500; choline chloride, 1200; and $B_{12}$, 0.040. Fat soluble vitamins were administered orally twice weekly so that the daily dosage was equivalent to 100 U.S.P. units of vitamin A acetate, 10 U.S.P. units of calciferol, and 1 mg. of alpha-tocopherol. The experimental rats (17 hypophysectomized) were injected subcutaneously 6 days a week for 4 weeks with 1.5 mg. of cortisone acetate ("Cortone," Merck) per dose diluted with saline to 0.1 ml. The control hypophysectomized animals were injected with equivalent amounts of physiological saline. After 4 weeks on the test a representative number of the surviving animals were killed by exsanguination under light ether anesthesia and the organs were removed, trimmed and weighed. Hypophysectomy was considered to have been proved adequate on the basis of signs of pituitary hypofunction; i.e., the growth of the animal was characteristically inhibited; target organs, notably the thyroid, the adrenals and the testes, were markedly atrophic, and spermatogenesis on histological examination of the testicles was found to be absent. The fat content of the livers was determined by extraction with hot alcohol-ether, followed by extraction with hot chloroform. The combined solvents were then evaporated and re-extracted with petroleum ether, dried over anhydrous sodium sulfate evaporated and determined gravimetrically.

## RESULTS AND DISCUSSION

Although the average food consumption, measured over the period of the last three weeks of the experiment, did not differ in the 2 groups (115 gm. in the controls compared with 110 gm. in the rats treated with cortisone), a significant difference was observed in the behavior of the body weights. The hypophysectomized rats gained an average of 15.4 gm. over the initial body weight. Cortisone, on the other hand, as has also been shown with ACTH (Asling *et al.*, 1951; Benzńak, 1952) led to an average loss in body weight of 17 gm. No gross differences in the appearance of the animals in the 2 groups could be detected; however, the tendency for the retention of the soft, infantile fur described by Asling *et al.* (1951) for hypophysectomized rats seemed still more marked in the animals treated with cortisone. The mortality rate of the hypophysectomized rats injected with cortisone was slightly, but not significantly, higher than that of their hypophysectomized controls. Exophthalmos, a common result of treatment of intact rats with cortisone (Aterman and Greenberg, 1953; Williams, 1953), was never observed in the hypophysectomized rats so treated.

Determination of the fat content of the livers (wet basis) in the 2 groups did not show any difference, the percentage of liver fat in the hypophysectomized controls (4.73) practically equalling that in the rats treated with cortisone (4.84). These findings differ from the results reported by Li, Simpson and Evans (1949) who noted that ACTH increased the fat content of the livers not only of intact rats but also of hypophysectomized rats. It is pertinent to point out in this connection that cortisone, given to intact rats in the same dose and over the same period of time but not in the same diet as in the present experiment, produced a striking increase in the fat content of the liver (Greenberg and Aterman, unpublished).

The absolute and relative organ weights of the cortisone-treated and of the control hypophysectomized animals are presented in Table 1. The data, to a large extent, bear out the findings obtained by Asling *et al.* (1951) by the use of ACTH. It is apparent that most of the effects of cortisone in the hypophysectomized rat, when compared with the effects in intact animals, are not dependent on the complete functional integrity of the pituitary gland. The smaller scale of the changes observed in some organs of the hypophysectomized animal, when treated with cortisone, is probably merely the result of its lowered metabolism. On the other hand, the failure of cortisone to raise the fat content of the liver and to produce exophthalmos suggests that hypophysectomy removes one or several factors essential for the production of these phenomena.

Under the influence of cortisone the absolute liver weight decreased from an average of $3308 \pm 133$ mg. to $2613 \pm 160$ mg. ($P < 0.01$), whereas the relative liver weight showed

TABLE 1. THE EFFECT OF CORTISONE ADMINISTRATION OVER A PERIOD OF 4 WEEKS ON THE ABSOLUTE AND RELATIVE[1] ORGAN WEIGHTS (IN PARENTHESES) OF HYPOPHYSECTOMIZED MALE RATS. EACH WEIGHT INCLUDES THE STANDARD ERROR OF THE MEAN

| Body weight | Liver | Heart (ventricle) | Kidneys | Adrenals | Spleen | Thymus | Testes | Seminal vesicles | Thyroid | Eyeballs |
|---|---|---|---|---|---|---|---|---|---|---|
| gm. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. | mg. |
| | | | | *Hypophysectomized Controls (6 rats)* | | | | | | |
| 79 ± 2.4 | 3308 ± 133<br>(4192 ± 193) | 383 ± 15<br>(486 ± 25) | 819 ± 29<br>(1040 ± 51) | 8.7 ± 0.7<br>(10.9 ± 0.8) | 262 ± 35<br>(333 ± 49) | 293 ± 61<br>(378 ± 88) | 143 ± 15<br>(180 ± 15) | 5.7 ± 0.5<br>(7.1 ± 0.5) | 4.8 ± 0.2<br>(6.0 ± 0.4) | 201 ± 4<br>(255 ± 11) |
| | | | | *Hypophysectomized-Cortisone Treated (9 rats)* | | | | | | |
| 52 ± 4.2 | 2613 ± 160<br>(5084 ± 242) | 313 ± 21<br>(607 ± 19) | 804 ± 47<br>(1562 ± 62) | 6.8 ± 0.4<br>(13.5 ± 1.2) | 141 ± 20<br>(276 ± 42) | 12 ± 8.2<br>(17 ± 11.2) | 148 ± 21<br>(279 ± 26) | 7.8 ± 0.7<br>(15.2 ± 1.3) | 3.7 ± 0.3<br>(7.5 ± 1.0) | 176 ± 4<br>(351 ± 22) |

[1] Relative organ weight = weight per 100 gm. body weight.

an increase ($P < 0.05$). As in the intact rat (Greenberg and Aterman, unpublished) cortisone produced in the hypophysectomized rat some decrease in the absolute ($P < 0.05$) and a marked increase in the relative weight ($P < 0.01$) of the heart. The absolute weight of the kidneys was practically unchanged, but the relative weight was considerably raised by the administration of cortisone. In the absence of direct measurements of the blood pressure it is not possible to ascertain whether the marked increase in the relative weights of the heart and of the kidneys, noted also by Asling *et al.* (1951) after treatment with ACTH, is due to a direct effect of cortisone on these organs, or whether it is the result of the increase in blood pressure which has been demonstrated in the intact rat by Paricenti and Sala (1951). Other workers, quoted by Bezńak (1952), have observed that adrenocortical steroids restore, at least partially, the blood pressure of the hypophysectomized rat.

The adrenals, the thymus and the spleen all showed a decrease of the absolute weights after treatment with cortisone. The adrenals show only a slight ($P < 0.05$) involution upon treatment of the hypophysectomized rat with cortisone; the relative adrenal weights of the experimental rats showed little unchange. The effect of cortisone on the thymus was as complete in the hypophysectomized as in the intact rat.

The testicles and the seminal vesicles of the hypophysectomized rats were markedly atrophic, and spermatogenesis was not observed. Cortisone had no effect on the absolute weight of the testicles but produced some increase in their relative weights ($P < 0.02$). Histological examination again did not show any evidence of spermatogenesis. The absolute weight of the seminal vesicles rose from an average of $5.7 \pm 0.5$ mg. in the hypophysectomized controls to $7.8 \pm 0.7$ mg. in the rats treated with cortisone ($P < 0.05$), but the relative weight doubled ($P < 0.001$) in the latter group. In the intact weanling male rat, cortisone, given in the same dose as in the present series, produced some decrease of the absolute, but a very striking increase in the relative weight of the testicles as a result of differential growth rates of tissues (Aterman and Greenberg, 1952). The development of the seminal vesicles in these animals was so clearly inhibited that an androgenic effect of cortisone in the intact rat could be ruled out. The findings in the hypophysectomized animal reported here are substantially in agreement with this latter conclusion. Although there is a significant increase in the relative seminal vesicle weight of the cortisone-treated, hypophysectomized rats, the degree of androgenic activity of the cortisone would appear to have no physiological importance. These observations lend support to the conclusions arrived at by Leroy (1951) and Courrier and Marois (1952) on the strength of other experiments, that cortisone has no appreciable androgenic properties.

Although the thyroid gland showed a decrease in the absolute weight under the influence of cortisone, the relative weight remained unchanged when compared with the controls. The data on the absolute and relative eyeball weights, although presented in Table 1, have already been reported elsewhere (Aterman and Greenberg, 1954) and will not be discussed here.

The liability of animals treated with cortisone to develop infections is well-known. The frequent presence of pus in the ear cavities of the hypophysectomized rats injected with this steroid was observed during the routine inspection of the pituitary fossa.

SUMMARY

The effect of prolonged treatment with cortisone on the body-weight and the organ weights of the immature hypophysectomized rat has been investigated. Cortisone does not alter the fat content of the liver in the hypophysectomized rat, nor does it produce ocular protrusion. These findings are compared with results obtained in intact rats treated with the same dose of cortisone, and with relevant findings in the literature.

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SAMUEL M. GREENBERG[5] AND K. ATERMAN

*Departments of Pharmacology and Anatomy*
*University of Birmingham Medical School*
*Birmingham, England*

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[5] Research Fellow, University of Birmingham. Present address: Biochemistry Section, Smith, Kline and French Laboratories, Philadelphia, Pa.

## FURTHER SPECIFICITY TESTS OF A BIO-ASSAY FOR PROGESTERONE[1]

A bio-assay for progesterone was described by Hooker and Forbes in 1947. The assay is based on the response of the stromal nuclei of the endometrium of the ovariectomized CHI mouse following the intrauterine injection of 0.0006–0.0008 cc. of sesame oil containing the ether- and acetone-soluble substances extracted from a measured volume of plasma, urine, or other biological material. Progressive dilutions are injected until the end point is reached. When standard procedure is observed, injection of oil containing 0.0002 μg. or more of progesterone into a ligated uterine segment results in 48 hours in a transformation of some of the previously shrunken stromal nuclei of the segment into nuclei which have (1) smooth, plump, elongated oval outlines, (2) conspicuous nucleoli, and (3) fine, evenly distributed chromatin particles. Extensive tests of the specificity of the response (Hooker and Forbes, 1947, 1949) have indicated that stromal nuclei meeting

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16-methoxyprogesterone and 16-dehydroprogesterone were kindly supplied by Dr. T. F. Gallagher of the Sloan-Kettering Institute for Cancer Research; $\Delta^{11}$-progesterone, by Dr. Albert Segaloff of the Alton Ochsner Medical Foundation; 4-hydroxyprogesterone, by Dr. Gregory Pincus of the Worcester Foundation for Experimental Biology; "Compound X," by Dr. W. H. Pearlman of Jefferson Medical College. Dr. K. G. Ober of the Universitäts-Frauenklinik, Marburg, Germany, generously sent 11-dehydroprogesterone he had received from Ciba-A.G., Wehr/Baden, Germany.

these rigid criteria are not found unless the uterine segments have been injected either with at least the minimal effective dose of progesterone or, questionably, with enormously greater quantities of certain other steroid compounds.

Specificity tests have now been made with nine additional substances: 4-hydroxyprogesterone, $\Delta^{11}$-progesterone, 11-dehydroprogesterone, 16-dehydroprogesterone, 16-methoxyprogesterone, cortisone acetate, 17-hydroxycorticosterone (Compound F), diethylstilbestrol, and Pearlman's "Compound X" (Pearlman and Cerceo, 1948). The following table indicates the amounts in micrograms which were injected into the uteri of test mice and the positive (+) or negative (−) response of the stromal nuclei.

TABLE 1. FURTHER SPECIFICITY TESTS OF COMPOUNDS IN BIO-ASSAY FOR PROGESTERONE

| | Intra-uterine injections, micrograms | |
|---|---|---|
| | — | + |
| Progesterone | 0.0001 | 0.0002 |
| 4-hydroxyprogesterone | 0.004, 0.0013, 0.0008 | |
| $\Delta^{11}$-progesterone | 3.072, 2.048, 1.538 | 6.114 |
| 11-dehydroprogesterone | 0.0008 (3 mice), 0.0004 (3 mice), 0.0002 (3 mice), 0.0001 (2 mice) | 0.0002 (1 horn) |
| 16-dehydroprogesterone | 17.04, 4.28 | |
| 16-methoxyprogesterone | 17.76, 4.44 | |
| Cortisone acetate | 4, 4 | |
| 17-hydroxycorticosterone | 0.4 (2 mice), 0.04 (2 mice), 0.004 (2 mice) | |
| Diethylstilbestrol | 3, 1.5, 0.3 | |
| Pearlman's "Compound X" | | 0.008, 0.004 |

The positive response to 6.144 $\mu$g. of $\Delta^{11}$-progesterone is not of practical significance, since half this amount (still a relatively huge quantity) had a negative effect. The positive response to 0.0002 $\mu$g. of 11-dehydroprogesterone of a uterine horn of a single mouse and the negative response of the horns of nine other mice to equal or higher doses of this compound would appear to indicate an aberrant reaction of the single horn rather than a failure of specificity.

The positive response to Pearlman's Compound X, a result already reported (Pearlman, 1952), seems, however, to represent a failure of the stromal nuclei to distinguish between this substance and progesterone. Compound X was isolated by Pearlman and Cerceo (1948) from the bile of pregnant cattle. These authors concluded that progesterone and Compound X were not identical. However, Pearlman and Cerceo did not identify Compound X; the extent to which it differs from progesterone, and the possible significance of the difference, remain unclear. It has been reported that sufficiently high estradiol-progesterone ratios may prevent a positive stromal nuclear response to progesterone (Olsen, Salhanick, and Hisaw, 1952). Such interference, however, does not seem to occur in mice receiving estrogen and progesterone in quantities and ratios within what is believed to be the physiological range (Zarrow and Neher, 1953). Endogenous progesterone is readily demonstrated in ovarian follicular fluid (Hooker and Forbes, 1947), where the estrogen concentration is presumed to be high. In addition, the positive response of the stromal nuclei was not interfered with when progesterone:pregnanediol mixtures and progesterone:testosterone mixtures in ratios as high as 1:500 were administered (Zarrow and Neher, 1953).

Using physico-chemical methods, Haskins (1950) failed to find any progesterone in peripheral blood samples from 10 women in unspecified stages of pregnancy. Butt,

Morris, Morris, and Williams (1951) did not detect the hormone in similar samples from 13 women in the twelfth to final week of pregnancy. Very recently, however, Zander (1954) who also employed physico-chemical procedures for the assay of 9 extracts prepared from a total of 46 peripheral blood samples obtained during the second half of human pregnancy, reported progesterone concentrations of 0.039–0.268 μg./cc. plasma. Application of the bio-assay (Forbes, 1951) to 48 peripheral blood samples drawn from two human donors at intervals of a week or oftener throughout most of the second and all of the third trimesters of pregnancy indicated that 11 samples contained less than detectable amounts of free progesterone, 10 samples contained 0.2 to 0.3 μg./cc. plasma, and 27 samples contained 0.4 to 2.3 μg./cc. plasma. The differences in progesterone levels reported by these investigators may be due simply to normal biological variation among donors. Alternatively, it is possible, as suggested by Butt *et al.* (1951) and by Edgar (1953), that the bio-assay may measure not only progesterone but some other compound as well. Assays of portions of the same plasma samples by biological and by physico-chemical methods should do much to resolve the question.

Individuals planning to use the bio-assay are urged to remember the following points. An inbred strain of mice, properly evaluated for specificity, sensitivity, and consistency of response, must be used as the source of test animals. Strain differences in sensitivity occur; the mice used as assay animals by the Marburg investigators (Klein and Ober, 1952), for example, respond to a minimal effective dose one-fourth of that required by mice of the CHI strain. In examining uterine sections for the progesterone effect, all stromal nuclei lying close to glands and blood vessels must be disregarded, as such nuclei frequently show a false positive response. No nucleus, even if at a distance from epithelium or endothelium, can be regarded as positive unless it unquestionably meets *all* three of the criteria described in the first paragraph.

THOMAS R. FORBES

*Department of Anatomy*
*Yale University*
*New Haven, Connecticut*

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# AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

## Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada.

## The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-four months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

## The Ayerst, McKenna and Harrison Fellowship

This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.

(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

### The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society established a category of Scholars. These grants have been made available through the generosity of The Schering Corporation and The Upjohn Company, and will be awarded to established investigators and teachers in the fields of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

### *Nominations*

Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1, 1954.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1954.